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(21) International Application Number: PCT/US99/01705 (22) International Filing Date: 27 January 1999 (27.01.99) (30) Priority Data: <table border="0"><tr><td>09/014,304</td><td>27 January 1998 (27.01.98)</td><td>US</td></tr><tr><td>60/073,011</td><td>29 January 1998 (29.01.98)</td><td>US</td></tr><tr><td>60/078,102</td><td>16 March 1998 (16.03.98)</td><td>US</td></tr><tr><td>60/084,425</td><td>6 May 1998 (06.05.98)</td><td>US</td></tr><tr><td>60/084,509</td><td>6 May 1998 (06.05.98)</td><td>US</td></tr><tr><td>09/135,183</td><td>17 August 1998 (17.08.98)</td><td>US</td></tr></table> (71) Applicant (for all designated States except US): CLINICAL MICRO SENSORS, INC. [US/US]; 101 Waverly Avenue, Pasadena, CA 91105 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): KAYYEM, Jon, Faiz [US/US]; 428 South Sierra Bonita Avenue, Pasadena, CA 91106 (US). (74) Agents: SILVA, Robin, M. et al.; Flehr, Hohbach, Test, Albritton & Herbert LLP, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		09/014,304	27 January 1998 (27.01.98)	US	60/073,011	29 January 1998 (29.01.98)	US	60/078,102	16 March 1998 (16.03.98)	US	60/084,425	6 May 1998 (06.05.98)	US	60/084,509	6 May 1998 (06.05.98)	US	09/135,183	17 August 1998 (17.08.98)	US	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
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(54) Title: AMPLIFICATION OF NUCLEIC ACIDS WITH ELECTRONIC DETECTION (57) Abstract The invention relates to compositions and methods useful in the detection of nucleic acids using a variety of amplification techniques, including both signal amplification and target amplification. Detection proceeds through the use of an electron transfer moiety (ETM) that is associated with the nucleic acid, either directly or indirectly, to allow electronic detection of the ETM using an electrode.																				

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AMPLIFICATION OF NUCLEIC ACIDS WITH ELECTRONIC DETECTION

The present invention is a continuing application of U.S.S.N.s 09/014,304, filed January 27, 1998; 60/073,011, filed January 29, 1998; 60/028,102, filed March 16, 1998; 60/084,425, filed May 6, 1998; 60/084,509, filed May 6, 1998; and 09/135,183, filed August 17, 1998, all of which are expressly
5 incorporated herein in their entirety.

FIELD OF THE INVENTION

The invention relates to compositions and methods useful in the detection of nucleic acids using a variety of amplification techniques, including both signal amplification and target amplification.

Detection proceeds through the use of an electron transfer moiety (ETM) that is associated with the
10 nucleic acid, either directly or indirectly, to allow electronic detection of the ETM using an electrode.

BACKGROUND OF THE INVENTION

The detection of specific nucleic acids is an important tool for diagnostic medicine and molecular biology research. Gene probe assays currently play roles in identifying infectious organisms such as bacteria and viruses, in probing the expression of normal genes and identifying mutant genes such as
15 oncogenes, in typing tissue for compatibility preceding tissue transplantation, in matching tissue or blood samples for forensic medicine, and for exploring homology among genes from different species.

Ideally, a gene probe assay should be sensitive, specific and easily automatable (for a review, see Nickerson, Current Opinion in Biotechnology 4:48-51 (1993)). The requirement for sensitivity (i.e. low detection limits) has been greatly alleviated by the development of the polymerase chain reaction
20 (PCR) and other amplification technologies which allow researchers to amplify exponentially a specific nucleic acid sequence before analysis as outlined below (for a review, see Abramson et al., Current Opinion in Biotechnology, 4:41-47 (1993)).

Sensitivity, i.e. detection limits, remain a significant obstacle in nucleic acid detection systems, and a variety of techniques have been developed to address this issue. Briefly, these techniques can be classified as either target amplification or signal amplification. Target amplification involves the amplification (i.e. replication) of the target sequence to be detected, resulting in a significant increase
5 in the number of target molecules. Target amplification strategies include the polymerase chain reaction (PCR), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA).

Alternatively, rather than amplify the target, alternate techniques use the target as a template to replicate a signalling probe, allowing a small number of target molecules to result in a large number of
10 signalling probes, that then can be detected. Signal amplification strategies include the ligase chain reaction (LCR), cycling probe technology (CPT), and the use of "amplification probes" such as "branched DNA" that result in multiple label probes binding to a single target sequence.

The polymerase chain reaction (PCR) is widely used and described, and involve the use of primer extension combined with thermal cycling to amplify a target sequence; see U.S. Patent Nos. 4,683,195
15 and 4,683,202, and PCR Essential Data, J. W. Wiley & sons, Ed. C.R. Newton, 1995, all of which are incorporated by reference.

Strand displacement amplification (SDA) is generally described in Walker et al., in Molecular Methods for Virus Detection, Academic Press, Inc., 1995, and U.S. Patent Nos. 5,455,166 and 5,130,238, all of which are hereby incorporated by reference.

20 Nucleic acid sequence based amplification (NASBA) is generally described in U.S. Patent No. 5,409,818 and "Profiting from Gene-based Diagnostics", CTB International Publishing Inc., N.J., 1996, both of which are incorporated by reference.

Cycling probe technology (CPT) is a nucleic acid detection system based on signal or probe amplification rather than target amplification, such as is done in polymerase chain reactions (PCR).
25 Cycling probe technology relies on a molar excess of labeled probe which contains a scissile linkage of RNA. Upon hybridization of the probe to the target, the resulting hybrid contains a portion of RNA:DNA. This area of RNA:DNA duplex is recognized by RNaseH and the RNA is excised, resulting in cleavage of the probe. The probe now consists of two smaller sequences which may be released, thus leaving the target intact for repeated rounds of the reaction. The unreacted probe is removed and
30 the label is then detected. CPT is generally described in U.S. Patent Nos. 5,011,769, 5,403,711, 5,660,988, and 4,876,187, and PCT published applications WO 95/05480, WO 95/1416, and WO 95/00667, all of which are specifically incorporated herein by reference.

The ligation chain reaction (LCR) involve the ligation of two smaller probes into a single long probe, using the target sequence as the template for the ligase. See generally U.S. Patent Nos. 5,185,243 and 5,573,907; EP 0 320 308 B1; EP 0 336 731 B1; EP 0 439 182 B1; WO 90/01069; WO 89/12696; and WO 89/09835, all of which are incorporated by reference.

5 "Branched DNA" signal amplification relies on the synthesis of branched nucleic acids, containing a multiplicity of nucleic acid "arms" that function to increase the amount of label that can be put onto one probe. This technology is generally described in U.S. Patent Nos. 5,681,702, 5,597,909, 5,545,730, 5,594,117, 5,591,584, 5,571,670, 5,580,731, 5,571,670, 5,591,584, 5,624,802, 5,635,352, 5,594,118, 5,359,100, 5,124,246 and 5,681,697, all of which are hereby incorporated by reference.

10 Similarly, dendrimers of nucleic acids serve to vastly increase the amount of label that can be added to a single molecule, using a similar idea but different compositions. This technology is as described in U.S. Patent No. 5,175,270 and Nilsen et al., J. Theor. Biol. 187:273 (1997), both of which are incorporated herein by reference.

15 Finally, U.S. Patent Nos. 5,591,578, 5,824,473, 5,770,369, 5,705,348, and 5,780,234, and PCT application WO98/20162 describe novel compositions comprising nucleic acids containing electron transfer moieties, including electrodes, which allow for novel detection methods of nucleic acid hybridization.

SUMMARY OF THE INVENTION

20 In accordance with the objects outlined above, the present invention provides methods for detecting a target nucleic acid sequence. The methods comprise hybridizing at least a first primer nucleic acid to the target sequence to form a first hybridization complex, and contacting the first hybridization complex with a first enzyme to form a modified first primer nucleic acid. The first hybridization complex is then disassociated. These steps may be repeated a plurality of times. A first assay complex is then formed comprising at least one ETM and the modified first primer nucleic acid. The assay complex is
25 covalently attached to an electrode. Electron transfer is then detected between the ETM and the electrode as an indication of the presence of the target sequence. The method can include the same method on a second target sequence substantially complementary to the the first target sequence.

In an additional aspect, the method utilizes a DNA polymerase and the modification to the primer is an extension of the primer such that the polymerase chain reaction (PCR) occurs.

In a further aspect, the method utilizes a ligase and the modification to the primer comprises a ligation of the first primer which hybridizes to a first domain of the first target sequence to a third primer which hybridizes to a second adjacent domain of the first target sequence, such that the ligase chain reaction (LCR) occurs.

- 5 In an additional aspect, the method utilizes a first primer comprising a first probe sequence, a first scissile linkage and a second probe sequence. The enzyme will cleave the first scissile linkage resulting in the separation of the first and the second probe sequences and the disassociation of the hybridization complex while leaving the first target sequence intact, such that the cycling probe technology (CPT) reaction occurs.
- 10 In a further aspect, the method utilizes a first enzyme that is a polymerase that extends the first primer to form a modified first primer comprising a first newly synthesized strand, and said method further comprises the addition of a second enzyme comprising a nicking enzyme that nicks the extended first primer leaving the first target sequence intact. The method additionally comprises extending from the nick using the polymerase, thereby displacing the first newly synthesized strand and generating a
- 15 second newly synthesized strand, such that strand displacement amplification (SDA) occurs.

- In an additional aspect, the method utilizes a first target sequence that is a RNA target sequence, a first primer nucleic acid that is a DNA primer comprising an RNA polymerase promoter, and the first enzyme is a reverse-transcriptase that extends the first primer to form a first newly synthesized DNA strand. The method further comprises the addition of a second enzyme comprising an RNA degrading
- 20 enzyme that degrades the first target sequence. A third primer is then added that hybridizes to the first newly synthesized DNA strand. A third enzyme is added comprising a DNA polymerase that extends the third primer to form a second newly synthesized DNA strand, to form a newly synthesized DNA hybrid. A fourth enzyme is then added comprising an RNA polymerase that recognizes the RNA polymerase promoter and generates at least one newly synthesized RNA strand from the DNA hybrid,
- 25 such that nucleic acid sequence-based amplification (NASBA) occurs.

- In a further aspect, the invention provides methods for detecting a target nucleic acid sequence comprising forming a first hybridization complex comprising an amplifier probe and a target sequence, wherein the amplifier probe comprises at least two amplification sequences and hybridizing a first
- 30 portion of at least one label probe to all or part of at least one amplification sequence. A second portion of the label probe is then hybridized to a detection probe covalently attached to an electrode via a conductive oligomer to form a second hybridization complex that contains at least a first electron transfer moiety (ETM). The label probe is then detected by measuring electron transfer between said first ETM and said electrode.

In an additional aspect, the invention provides methods for detecting a target nucleic acid sequence comprising forming a first hybridization complex comprising an amplifier probe and a target sequence, wherein the amplifier probe comprises at least two amplification sequences and wherein the first hybridization complex is covalently attached to an electrode comprising a monolayer comprising
5 conductive oligomers. At least one label probe comprising at least one electron transfer moiety (ETM) is hybridized to all or part of at least one amplification sequence, and the label probe is detected by measuring electron transfer between said first ETM and said electrode.

In a further aspect, the invention provides kits for the detection of a first target nucleic acid sequence comprising at least a first nucleic acid primer substantially complementary to at least a first domain of
10 the target sequence and at least a first enzyme that will modify the first nucleic acid primer. The kits additionally comprise an electrode comprising at least one detection probe covalently attached to the electrode via a conductive oligomer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1O depict a number of different compositions of the invention; the results are shown
15 in Example 1 and 2. Figure 1A depicts I, also referred to as **P290**. Figure 1B depicts II, also referred to as **P291**. Figure 1C depicts III, also referred to as **W31**. Figure 1D depicts IV, also referred to as **N6**. Figure 1E depicts V, also referred to as **P292**. Figure 1F depicts II, also referred to as **C23**. Figure 1G depicts VII, also referred to as **C15**. Figure 1H depicts VIII, also referred to as **C95**. Figure 1I depicts **Y63**. Figure 1J depicts another compound of the invention. Figure 1K depicts **N11**. Figure
20 1L depicts **C131**, with a phosphoramidite group and a DMT protecting group. Figure 1M depicts **W38**, also with a phosphoramidite group and a DMT protecting group. Figure 1N depicts the commercially available moiety that enables "branching" to occur, as its incorporation into a growing oligonucleotide chain results in addition at both the DMT protected oxygens. Figure 1O depicts **glen**, also with a phosphoramidite group and a DMT protecting group, that serves as a non-nucleic acid linker. Figures
25 1A to 1G and 1J are shown without the phosphoramidite and protecting groups (i.e. DMT) that are readily added.

Figure 2 depicts the synthetic scheme of a preferred attachment of an ETM, in this case ferrocene, to a nucleoside (in this case adenosine) via an oxo linkage to the ribose, forming the **N6** compound of the invention.

30 Figure 3 is similar to Figure 2 except that the nucleoside is cytidine, forming the **W38** compound of the invention.

Figure 4 depicts the synthetic scheme of a preferred attachment of an ETM, in this case ferrocene, to a nucleoside via the phosphate, forming the Y63 compound of the invention.

Figure 5 depicts the synthetic scheme of a triphosphate nucleotide, in this case adenosine, with an attached ETM, in this case ferrocene, via an oxo linkage to the ribose.

- 5 Figure 6 depicts the use of an activated carboxylate for the addition of a nucleic acid functionalized with a primary amine to a pre-formed SAM.

Figure 7 depicts a schematic of the use of "universal" type gene chips, utilizing restriction endonuclease sites.

- 10 Figures 8A and 8B depicts two phosphate attachments of conductive oligomers that can be used to add the conductive oligomers at the 5' position, or any position.

Figure 9 depicts the synthesis of an insulator (C109) to the ribose of a nucleoside for attachment to an electrode.

Figure 10 depicts the synthetic scheme of ethylene glycol terminated conductive oligomers.

- 15 Figures 11A, 11B and 11C depict the synthesis of three different "branch" points (in this case each using adenosine as the base), to allow the addition of ETM polymers. Figure 11A depicts the synthesis of the N17 compound of the invention. Figure 11B depicts the synthesis of the W90 compound, and Figure 11C depicts the synthesis of the N38 compound.

Figure 12 depicts a schematic of the synthesis of simultaneous incorporation of multiple ETMs into a nucleic acid, using the N17 "branch" point nucleoside.

- 20 Figure 13 depicts a schematic of an alternate method of adding large numbers of ETMs simultaneously to a nucleic acid using a "branch" point phosphoramidite, in this case utilizing three branch points (although two branch points are also possible; see for example Figure 1N) as is known in the art. As will be appreciated by those in the art, each end point can contain any number of ETMs.

- 25 Figure 14 shows a representative hairpin structure. 500 is a target binding sequence, 510 is a loop sequence, 520 is a self-complementary region, 530 is substantially complementary to a detection probe, and 530 is the "sticky end", that is, a portion that does not hybridize to any other portion of the probe, that contains the ETMs.

Figures 15A, 15B and 15C depict three preferred embodiments for attaching a target sequence to the electrode. Figure 15A depicts a target sequence 120 hybridized to a capture probe 100 linked via an attachment linker 106, which as outlined herein may be either a conductive oligomer or an insulator. The electrode 105 comprises a monolayer of passivation agent 107, which can comprise conductive oligomers (herein depicted as 108) and/or insulators (herein depicted as 109). As for all the embodiments depicted in the figures, n is an integer of at least 1, although as will be appreciated by those in the art, the system may not utilize a capture probe at all (i.e. n is zero), although this is generally not preferred. The upper limit of n will depend on the length of the target sequence and the required sensitivity. Figure 15B depicts the use of a single capture extender probe 110 with a first portion 111 that will hybridize to a first portion of the target sequence 120 and a second portion that will hybridize to the capture probe 100. Figure 15C depicts the use of two capture extender probes 110 and 130. The first capture extender probe 110 has a first portion 111 that will hybridize to a first portion of the target sequence 120 and a second portion 112 that will hybridize to a first portion 102 of the capture probe 100. The second capture extender probe 130 has a first portion 132 that will hybridize to a second portion of the target sequence 120 and a second portion 131 that will hybridize to a second portion 101 of the capture probe 100. As will be appreciated by those in the art, any of these attachment configurations may be used with any of the other systems, including the embodiments of Figure 16.

Figures 16A, 16B, 16C, 16D, 16E, 16F and 16G depict some of the embodiments of the invention. All of the monolayers depicted herein show the presence of both conductive oligomers 108 and insulators 107 in roughly a 1:1 ratio, although as discussed herein, a variety of different ratios may be used, or the insulator may be completely absent. In addition, as will be appreciated by those in the art, any one of these structures may be repeated for a particular target sequence; that is, for long target sequences, there may be multiple assay complexes formed. Additionally, any of the electrode-attachment embodiments of Figure 15 may be used in any of these systems.

Figures 16A, 16B and 16D have the target sequence 120 containing the ETMs 135; as discussed herein, these may be added enzymatically, for example during a PCR reaction using nucleotides modified with ETMs, resulting in essentially random incorporation throughout the target sequence, or added to the terminus of the target sequence. Figure 16C depicts the use of two different capture probes 100 and 100', that hybridize to different portions of the target sequence 120. As will be appreciated by those in the art, the 5'-3' orientation of the two capture probes in this embodiment is different.

Figure 16C depicts the use of label probes **145** that hybridize directly to the target sequence **120**. Figure 16C shows the use of a label probe **145**, comprising a first portion **141** that hybridizes to a portion of the target sequence **120**, a second portion **142** comprising ETMs **135**.

Figures 16E, 16F and 16G depict systems utilizing label probes **145** that do not hybridize directly to the target, but rather to amplifier probes that are directly (Figure 16E) or indirectly (Figures 16F and 16G) hybridized to the target sequence. Figure 16E utilizes an amplifier probe **150** has a first portion **151** that hybridizes to the target sequence **120** and at least one second portion **152**, i.e. the amplifier sequence, that hybridizes to the first portion **141** of the label probe. Figure 16F is similar, except that a first label extender probe **160** is used, comprising a first portion **161** that hybridizes to the target sequence **120** and a second portion **162** that hybridizes to a first portion **151** of amplifier probe **150**. A second portion **152** of the amplifier probe **150** hybridizes to a first portion **141** of the label probe **140**, which also comprises a recruitment linker **142** comprising ETMs **135**. Figure 16G adds a second label extender probe **170**, with a first portion **171** that hybridizes to a portion of the target sequence **120** and a second portion that hybridizes to a portion of the amplifier probe.

Figure 16H depicts a system that utilizes multiple label probes. The first portion **141** of the label probe **140** can hybridize to all or part of the recruitment linker **142**.

Figures 17A, 17B, 17C, 17D and 17E depict different possible configurations of label probes and attachments of ETMs. In Figures 17A-C, the recruitment linker is nucleic acid; in Figures 17D and E, it is not. A = nucleoside replacement; B = attachment to a base; C = attachment to a ribose; D = attachment to a phosphate; E = metallocene polymer (although as described herein, this can be a polymer of other ETMs as well), attached to a base, ribose or phosphate (or other backbone analogs); F = dendrimer structure, attached via a base, ribose or phosphate (or other backbone analogs); G = attachment via a "branching" structure, through base, ribose or phosphate (or other backbone analogs); H = attachment of metallocene (or other ETM) polymers; I = attachment via a dendrimer structure; J = attachment using standard linkers.

Figure 18 depicts an improvement utilizing a stem-loop probe. This can be desirable as it creates torsional strain on the surface-bound probe, which has been shown to increase binding efficiency and in some cases thermodynamic stability. In this case, the surface bound probe comprises a capture probe **100**, a first stem-loop sequence **550**, a target binding sequence **560**, and a second stem-loop sequence **570** that is substantially complementary to the first stem-loop sequence. Upon addition of the target sequence **120**, which can contain the ETMs **135** either directly or indirectly using a label probe **145**, the effective concentration of the target at the surface increases.

Figures 19A-19AA depict some of the sequences used in Example 1.

Figures 20A - 20O depict representative scans from the experiments outlined in Example 1. Unless otherwise noted, all scans were run at initial voltage -0.11 V, final voltage 0.5 V, with points taken every 10 mV, amplitude of 0.025, frequency of 10 Hz, a sample period of 1 sec, a quiet time of 2 sec.

- 5 Figure 20A has a peak potential of 0.160 V, a peak current of 1.092×10^{-8} A, and a peak A of 7.563×10^{-10} VA. Figure 20C has a peak potential of 0.190 V, a peak current of 2.046×10^{-7} A, and a peak area of 2.046×10^{-8} VA. Figure 20d has a peak potential of 0.190 V, a peak current of 3.552×10^{-8} A, and a peak A of 3.568×10^{-9} VA. Figure 20E has a peak potential of 0.190 V, a peak current of 2.3762×10^{-7} A, and a peak area of 2.594×10^{-8} VA. Figure 20F has a peak potential of 0.180 V, a peak
10 current of 2.992×10^{-8} A, and a peak area of 2.709×10^{-9} VA. Figure 20G has a peak potential of 0.150 V, a peak current of 1.494×10^{-7} A, and a peak area of 1.1×10^{-8} VA. Figure 20H has a peak potential of 0.160 V, a peak current of 1.967×10^{-8} A, and a peak area of 1.443×10^{-9} VA. Figure 20I has a peak potential of 0.150 V, a peak current of 8.031×10^{-8} A, and a peak area of 6.033×10^{-9} VA. Figure 20J has a peak potential of 0.150 V, a peak current of 8.871×10^{-9} A, and a peak area of 5.51×10^{-10} VA. Figure 20L has a peak potential of 0.140 V, a peak current of 2.449×10^{-8} A, and a peak
15 area of 1.706×10^{-9} VA. Figure 20M has a peak potential of 0.150 V, a peak current of 6.637×10^{-8} A, and a peak area of 7.335×10^{-9} VA. Figure 20N has a peak potential of 0.140 V, a peak current of 2.877×10^{-8} A, and a peak area of 2.056×10^{-10} VA.

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Figure 21 depicts the ligation chain reaction (LCR) experiment of Example 13.

- 20 Figures 22A and 22B depicts the results of Example 12. The "hybrid code" refers to the system number; + and - refer to the presence or absence of the rRNA target.

Figures 23A, 23B, 23C, 23D, 23E and 23F depict the compositions and results of Example 13.

Figures 24A and 24B depict the compositions and results from Example 13.

Figures 25A and 25B depict the set up of two of the experiments of Example 8.

- 25 Figure 26 shows the results of a PCR experiment as outlined in Example 9.

Figures 27A, 27B, 27C, 27D, 27E and 27F depict some "mechanism-1" detection systems. Figure 27A shows portions of target sequence 120 hybridized to detection probes 300 linked via conductive oligomers 108 to electrode 105. The hybridization complex comprises an ETM 135 that can be covalently linked either to the target sequence or the detection probe 300, or non-covalently (i.e. a

hybridization indicator). The monolayer is depicted with insulators 107, although as outlined herein, any type of passivation agent may be used. Figure 27B depicts the use of capture probe 100 linked via attachment linker 106 to electrode 105, although as will be appreciated, since the label probe 145 hybridizes to the detection probe 300 this can serve as a type of capture probe. A label probe 145

5 comprising a first portion 141 that hybridizes to a portion of the target sequence 120 and a second portion 142 that hybridizes to the detection probe 300. Figure 27C depicts the use of branched amplifier probe 150 comprising a first portion 151 that hybridizes to the target sequence 120 (although label extender probes can be used as well) and amplification sequences 152 that hybridize directly to the detection probes 300. The target sequence may be additionally attached to the electrode using

10 capture probes as outlined in Figure 15. Figure 27D depicts the same thing utilizing a linear amplification probe 150. Figure 27E depicts a similar system, but uses label probes 145 comprising a first portion 141 that hybridizes to the amplification sequence 152 and a second portion 142 that hybridizes to a detection probe 300. Figure 27F depicts the same thing but using a linear amplification probe 150.

15 Figure 28 depicts an LCR embodiment of the invention. A first probe nucleic acid 200 and a second probe nucleic acid 210 are hybridized to a first and second target domains of a single-stranded target sequence 120. The probes are attached, generally through ligation, to form a ligated probe 220. The first hybridization complex 225 is denatured, and the process is repeated, to generate a pool of

20 ligated probes 220. The unligated probes 200 and 210 are removed as is known in the art, and then the ligated probes 220 are hybridized to detection probes 300 and detected as outlined herein. As will be appreciated by those in the art, the target domains depicted are directly adjacent, i.e. contiguous, but gaps that are then filled using nucleotides and polymerase can also be used. In addition, the probes are depicted with attached ETMs 135, although as will be appreciated, non-covalently attached ETMs may also be used.

25 Figure 29 depicts an alternate LCR embodiment of the invention. A first probe nucleic acid 200 and a second probe nucleic acid 210 are hybridized to a first and second target domains of a single-stranded target sequence 120. A third probe nucleic acid 200 and a fourth probe nucleic acid 210 are hybridized to a third and fourth target domains of the complementary single-stranded target sequence

30 125. The probes are attached, generally through ligation, to form ligated probes 220 and 221. The hybridization complexes 225 and 226 are denatured, and the process is repeated to generate a pool of ligated probes 220 and 221. The unligated probes 200, 201, 210 and 211 are removed as is known in the art, and then the ligated probes 220 and 221 are hybridized to detection probes 300 and detected as outlined herein. As will be appreciated by those in the art, the target domains depicted are directly adjacent, i.e. contiguous, but gaps that are then filled using nucleotides and polymerase can also be

used. In addition, the probes are depicted with attached ETMs, although as will be appreciated, non-covalently attached ETMs may also be used.

Figure 30 depicts a preferred CPT embodiment of the invention; Figure 30 depicts a "mechanism-1" system, but as will be appreciated by those in the art, "mechanism-2" systems may be used as well. A primary probe 1, comprising a first probe sequence 10, a scissile linkage 11, and a second probe sequence 12, with two covalently attached ETMs 13, is added to a target sequence 120, to form a hybridization complex, 6. While Figure 30 depicts two ETMs, only one of the probe sequences may be covalently labeled with an ETM. Furthermore, additional probe sequences, and additional scissile linkages, may also be used. Upon subjection to cleavage conditions, the scissile linkage 11 is cleaved, leaving the two probe sequences 10 and 12 hybridized to the target sequence 120. These probe sequences then disassociate, leaving the probe sequences 10 and 12, and the target sequence 120. The target sequence is then free to hybridize with additional primary probes 1, and the reaction is repeated, generating a pool of probe sequences. The uncleaved primary probes are removed as outlined herein, and the pool of probe sequences is added to an electrode 105, with an optional layer of passivation agent 107, and detection probes 300 and 302 covalently attached via conductive oligomers 108. The detection probes and the probe sequences form a hybridization complex, 30. The detection probes may be mixed on the electrode, or may be at separate addresses 101 and 102. Additional ETMs in the form of hybridization indicators may optionally be added. Electron transfer is then initiated as is outlined herein. In addition, while this figure depicts a soluble reaction, the primary probes may be bound to a solid support as is described herein.

Figure 31 depicts an additional CPT embodiment utilizing a single capture sequence. A primary probe 1, comprising a first capture sequence 14, first probe sequence 10, a scissile linkage 11, and a second probe sequence 12, with a covalently attached ETM 135, is added to a target sequence 124, to form a hybridization complex, 6. Preferably, the capture sequence 14 does not hybridize to the target, although it can. While Figure 31 depicts only one covalently attached ETM, the other probe sequence 12 may be covalently labeled with an ETM. Furthermore, additional probe sequences, and additional scissile linkages, may also be used. Upon subjection to cleavage conditions, the scissile linkage 11 is cleaved, leaving the two probe sequences 10 and 12 hybridized to the target sequence 120. These probe sequences then disassociate, leaving the probe sequences 14-10 and 12, and the target sequence 5. The target sequence is then free to hybridize with additional primary probes 1, and the reaction is repeated, generating a pool of probe sequences. The uncleaved primary probes are removed as outlined herein, and the pool of probe sequences is added to an electrode 105, with an optional layer of passivation agent 107, and a detection probe, comprising the substantial complement of the capture sequence 24 and the substantial complement of the first probe sequence 20, covalently attached via conductive oligomers 108. Also depicted are detection probes 304 that only comprise

probes for the capture sequence. The detection probes and the probe sequences form a hybridization complex, 30. Additional ETMs in the form of hybridization indicators may optionally be added. Electron transfer is then initiated as is outlined herein. In addition, while this figure depicts a soluble reaction, the primary probes may be bound to a solid support as is described herein.

- 5 Figure 32 depicts the use of two capture sequences in a CPT application. A primary probe 1, comprising a first capture sequence 14, a first probe sequence 10, a scissile linkage 11, a second probe sequence 12, and a second capture sequence 15, with two covalently attached ETMs 135, is added to a target sequence 120, to form a hybridization complex, 6. Preferably, the capture sequence 14 does not hybridize to the target, although it can. While Figure 32 depicts two covalently attached
- 10 ETMs, there may be only one or none. Furthermore, additional probe sequences, and additional scissile linkages, may also be used. Upon subsection to cleavage conditions, the scissile linkage 11 is cleaved, leaving the two probe sequences with associated capture sequences, 14-10 and 12-15 hybridized to the target sequence 120. These probe sequences then disassociate, leaving the probe/capture sequences 14-10 and 12-15, and the target sequence 120. The target sequence is
- 15 then free to hybridize with additional primary probes 1, and the reaction is repeated, generating a pool of probe sequences. The uncleaved primary probes is neutralized by the addition of a substantially complementary neutralization probe 40, comprising sequences that are the substantial complement of the first capture sequence 24, the first probe sequence 20, the scissile linkage 25, the second probe sequence 22, and the second capture sequence 26. As will be appreciated by those in the art, the
- 20 complementary scissile linkage 25 is only required in those embodiments that utilize nucleic acid scissile linkages. As is depicted, the neutralization probe 40 may also be bound to the electrode. The pool of probe sequences is added to an electrode 105, with an optional layer of passivation agent 107, and detection probes, comprising the substantial complement of the capture sequence 24 and the substantial complement of the first probe sequence 20, and the substantial complement of the capture
- 25 sequence 22 and the substantial complement of the first probe sequence 26, covalently attached via conductive oligomers 108. The detection probes and the probe sequences form a hybridization complex, 30. Additional ETMs in the form of hybridization indicators may optionally be added. Electron transfer is then initiated as is outlined herein. In addition, while this figure depicts a soluble reaction, the primary probes may be bound to a solid support as is described herein.
- 30 Figure 33 depicts a similar reaction, where a separation sequence 210 is used to remove the uncleaved scissile probe, in this case a primary probe 1. A primary probe 1, comprising a first probe sequence 10, a first scissile linkage 11, a separation sequence 210, a second scissile linkage 11, and a second probe sequence 12, with two covalently attached ETMs 13, is added to a target sequence 120, to form a hybridization complex, 6. Other configurations include those depicted. While Figure 33
- 35 depicts two ETMs, only one of the probe sequences may be covalently labeled with an ETM.

Furthermore, additional probe sequences, and additional scissile linkages, may also be used. Upon
subjection to cleavage conditions, the scissile linkage **11** is cleaved, leaving the two probe sequences
10 and **12** and the separation sequence **210** hybridized to the target sequence **120**. In alternate
embodiments, the separation sequence does not hybridize to the target, for example when it is at a
terminus of the probe; this may be preferred as it allows generic "separation beads" to be used with
any target. These probe sequences then disassociate, leaving the probe sequences **10** and **12**, and
separation sequence **210**, and the target sequence **120**. The target sequence is then free to
hybridize with additional primary probes **1**, and the reaction is repeated, generating a pool of probe
sequences. The uncleaved primary probes are removed by the addition of a solid support bead **200**,
with the substantial complement of the separation sequence **212** attached, generally via a linker. This
bead then binds up the uncleaved probe **1** and the cleaved separation sequences **210**, leaving only
cleaved probe sequences in solution. The pool of probe sequences is added to an electrode **105**, with
an optional layer of passivation agent **107**, and detection probes **300** and **302** covalently attached via
conductive oligomers **108**. The detection probes and the probe sequences form a hybridization
complex, **30**. The detection probes may be mixed on the electrode, or may be at separate addresses
101 and **102**. Additional ETMs in the form of hybridization indicators may optionally be added.
Electron transfer is then initiated as is outlined herein.

Figure 34 depicts the use of solid-support bound primary scissile probes. A solid support bead **200**
with attached primary probes **1** is added to the target sequence **120**, to form a hybridization complex,
6. The primary probes may comprise an optional additional sequence **16** (which may be used to
stabilize the first scissile linkage hybrid, if necessary), a first scissile linkage **11**, a first probe sequence
10, a second scissile linkage **11**, and a second probe sequence **12**. While Figure 34 does not utilize
covalently attached ETMs, one or more of the probe sequences may be so labelled. Furthermore,
additional probe sequences, and additional scissile linkages, may also be used. Upon subjection to
cleavage conditions, the scissile linkage **11** is cleaved, leaving the two probe sequences **10** and **12**
hybridized to the target sequence **120**. These probe sequences then disassociate, leaving the probe
sequences **10** and **12**, and the target sequence **120**. The target sequence is then free to hybridize
with additional primary probes **1**, and the reaction is repeated, generating a pool of probe sequences.
The uncleaved primary probes are removed by removing the beads, and the pool of probe sequences
is added to an electrode **105**, with an optional layer of passivation agent **107**, and detection probes
300 and **302** covalently attached via conductive oligomers **108**. The detection probes and the probe
sequences form a hybridization complex, **30**. The detection probes may be mixed on the electrode, or
may be at separate addresses **101** and **102**. ETMs in the form of hybridization indicators are then
added, and electron transfer is then initiated as is outlined herein.

Figure 35 depicts the use of bead-bound primary and secondary probes. Two (or more) types of beads are used. The first type is a solid support bead 250 with attached secondary scissile probes 2 comprising a first scissile linkage 11, a first secondary probe sequence 60, a second scissile linkage 11, and a second secondary probe sequence 70. In addition, as depicted, a second secondary scissile probe may be preferably used, comprising a first scissile linkage 11, a first secondary probe sequence 80, a second scissile linkage 11, and a second secondary probe sequence 90. These are depicted in Figure 35 as being on the same bead, although two sets of beads may be preferably used. The primary probe beads 260 have attached primary probes 1 comprising a first scissile linkage 11, a first probe sequence 10, a second scissile linkage 11, and a second probe sequence 12. As outlined above, the probes may contain optional additional sequences (depicted herein as 16), or additional probe sequences or scissile linkages. The beads 250 and 260 are added to the target sequence 120. The primary probes form a hybridization complex, 6, with the target 120. While Figure 35 does not utilize covalently attached ETMs, any or all of the probe sequences may be so labelled. Upon subsection to cleavage conditions, the scissile linkages 11 are cleaved, leaving the two primary probe sequences 10 and 12 hybridized to the target sequence 120. These probe sequences then disassociate, leaving the probe sequences 10 and 12, and the target sequence 120. The target sequence is then free to hybridize with additional primary probes 1, and the reaction is repeated, generating a pool of probe sequences. The primary probe sequences are then free to diffuse to the secondary beads 250, where they may serve as the next "target", hybridizing to the secondary probes to form additional hybridization complexes, 7 and 8. Cleavage, followed by secondary probe sequence disassociation from the primary probe sequence "targets", generates a pool of secondary probe sequences which can be detected. The uncleaved probes are removed by removing the beads, and the pool of probe sequences is added to an electrode 105, with an optional layer of passivation agent 107, and detection probes 62, 72, 82, and 92 covalently attached via conductive oligomers 108. While Figure 35 does not show this, there may be detection probes for the primary probe sequences as well. The detection probes and the probe sequences form hybridization complexes. The detection probes may be mixed on the electrode, or may be at separate addresses. ETMs in the form of hybridization indicators are then added, and electron transfer is then initiated as is outlined herein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to compositions and methods useful in the detection of nucleic acids using a variety of amplification techniques, including both signal amplification and target amplification. Once amplification has occurred, detection proceeds based on electron transfer, as is described below and generally outlined in U.S. Patent Nos. 5,591,578, 5,824,473, 5,770,369, 5,705,348, and 5,780,234, and PCT application WO98/20162, all of which are expressly incorporated herein by reference in their entirety.

Accordingly, in a preferred embodiment, the present invention provides methods of detecting target nucleic acids utilizing amplification. By "target nucleic acid" or "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. In some embodiments, it may be desirable to fragment or cleave the sample nucleic acid into fragments of 100 to 10,000 basepairs, with fragments of roughly 500 basepairs being preferred in some embodiments. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. The sample comprising the target sequence may be virtually any tissue from any organism, including blood, bone marrow, lymph, hard tissues (e.g. organs such as liver, spleen, kidney, heart, lung, etc.) saliva, vaginal and anal secretions, urine, feces, perspiration, tears, and other bodily fluids, as well as cell lysates of bacteria and pathogens, including viruses.

As is outlined more fully below, probes (including primers) are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

The target sequence may also be comprised of different target domains; for example, in "sandwich" type assays as outlined below, a first target domain of the sample target sequence may hybridize to a capture probe or a portion of capture extender probe, a second target domain may hybridize to a portion of an amplifier probe, a label probe, or a different capture or capture extender probe, etc. In addition, the target domains may be adjacent (i.e. contiguous) or separated. For example, when LCR techniques are used, a first primer may hybridize to a first target domain and a second primer may hybridize to a second target domain; either the domains are adjacent, or they may be separated by one or more nucleotides, coupled with the use of a polymerase and dNTPs, as is more fully outlined below.

The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target sequence. For example, assuming a 5'-3' orientation of the complementary target sequence, the first target domain may be located either 5' to the second domain, or 3' to the second domain.

If required, the target sequence is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, sonication, electroporation, etc., with purification occurring as needed, as will be appreciated by those in the art. In addition, the reactions outlined

herein may be accomplished in a variety of ways, as will be appreciated by those in the art.

Components of the reaction may be added simultaneously, or sequentially, in any order, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used, depending on the sample preparation methods and purity of the target.

In addition, in most embodiments, double stranded target nucleic acids are denatured to render them single stranded so as to permit hybridization of the primers and other probes of the invention. A preferred embodiment utilizes a thermal step, generally by raising the temperature of the reaction to about 95°C, although pH changes and other techniques may also be used.

A primer nucleic acid is then contacted to the target sequence to form a hybridization complex. By "primer nucleic acid" herein is meant a probe nucleic acid that will hybridize to some portion, i.e. a domain, of the target sequence. Probes of the present invention are designed to be complementary to a target sequence (either the target sequence of the sample or to other probe sequences, as is described below), such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions.

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions; see for example Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al, hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH and nucleic acid

concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

Thus, the assays are generally run under stringency conditions which allows formation of the hybridization complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration pH, organic solvent concentration, etc.

These parameters may also be used to control non-specific binding, as is generally outlined in U.S. Patent No. 5,681,697. Thus it may be desirable to perform certain steps at higher stringency conditions to reduce non-specific binding.

The probes (including primers) comprise nucleic acids. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 9(1986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowski et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger

et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of ETMs, or to increase the stability and half-life of such molecules in physiological environments. In addition, it should be noted that the use of the terms "DNA" and "RNA" include nucleic acid analogs.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for example, at the site of conductive oligomer or ETM attachment, an analog structure may be used. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (T_m) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4°C drop in T_m for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9°C. This allows for better detection of mismatches. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. This is particularly advantageous in the systems of the present invention, as a reduced salt hybridization solution has a lower Faradaic current than a physiological salt solution (in the range of 150 mM).

The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribonucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc. A preferred embodiment utilizes

isocytosine and isoguanine in nucleic acids designed to be complementary to other probes, rather than target sequences, as this reduces non-specific hybridization, as is generally described in U.S. Patent No. 5,681,702. As used herein, the term "nucleoside" includes nucleotides as well as nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

The size of the primer nucleic acid may vary, as will be appreciated by those in the art, in general varying from 5 to 500 nucleotides in length, with primers of between 10 and 100 being preferred, between 15 and 50 being particularly preferred, and from 10 to 35 being especially preferred, depending on the use and amplification technique.

In addition, the different amplification techniques may have further requirements of the primers, as is more fully described below.

Once the hybridization complex between the primer and the target sequence has been formed, an enzyme, sometimes termed an "amplification enzyme", is used to modify the primer. As for all the methods outlined herein, the enzymes may be added at any point during the assay, either prior to, during, or after the addition of the primers. The identification of the enzyme will depend on the amplification technique used, as is more fully outlined below. Similarly, the modification will depend on the amplification technique, as outlined below, although generally the first step of all the reactions herein is an extension of the primer, that is, nucleotides are added to the primer to extend its length.

Once the enzyme has modified the primer to form a modified primer, the hybridization complex is disassociated. Generally, the amplification steps are repeated for a period of time to allow a number of cycles, depending on the number of copies of the original target sequence and the sensitivity of detection, with cycles ranging from 1 to thousands, with from 10 to 100 cycles being preferred and from 20 to 50 cycles being especially preferred.

After a suitable time or amplification, the modified primer is incorporated into an assay complex, as is more fully outlined below. The assay complex is covalently attached to an electrode, and comprises at least one electron transfer moiety (ETM), described below. Electron transfer between the ETM and the electrode is then detected to indicate the presence or absence of the original target sequence, as described below.

5 In a preferred embodiment, the amplification is target amplification. Target amplification involves the amplification (replication) of the target sequence to be detected, such that the number of copies of the target sequence is increased. Suitable target amplification techniques include, but are not limited to, the polymerase chain reaction (PCR), strand displacement amplification (SDA), and nucleic acid sequence based amplification (NASBA).

10 In a preferred embodiment, the target amplification technique is PCR. The polymerase chain reaction (PCR) is widely used and described, and involve the use of primer extension combined with thermal cycling to amplify a target sequence; see U.S. Patent Nos. 4,683,195 and 4,683,202, and PCR Essential Data, J. W. Wiley & sons, Ed. C.R. Newton, 1995, all of which are incorporated by reference. In addition, there are a number of variations of PCR which also find use in the invention, including "quantitative competitive PCR" or "QC-PCR", "arbitrarily primed PCR" or "AP-PCR", "immuno-PCR", "Alu-PCR", "PCR single strand conformational polymorphism" or "PCR-SSCP", "reverse transcriptase PCR" or "RT-PCR", "biotin capture PCR", "vectorette PCR", "panhandle PCR", and "PCR select cDNA subtration", among others. In one embodiment, the amplification technique is not PCR.

15 In general, PCR may be briefly described as follows. A double stranded target nucleic acid is denatured, generally by raising the temperature, and then cooled in the presence of an excess of a PCR primer, which then hybridizes to the first target strand. A DNA polymerase then acts to extend the primer, resulting in the synthesis of a new strand forming a hybridization complex. The sample is then heated again, to disassociate the hybridization complex, and the process is repeated. By using a second PCR primer for the complementary target strand, rapid and exponential amplification occurs. Thus PCR steps are denaturation, annealing and extension. The particulars of PCR are well known, and include the use of a thermostabile polymerase such as Taq I polymerase and thermal cycling.

Accordingly, the PCR reaction requires at least one PCR primer and a polymerase.

25 In a preferred embodiment, the target amplification technique is SDA. Strand displacement amplification (SDA) is generally described in Walker et al., in Molecular Methods for Virus Detection, Academic Press, Inc., 1995, and U.S. Patent Nos. 5,455,166 and 5,130,238, all of which are hereby expressly incorporated by reference in their entirety.

30 In general, SDA may be described as follows. A single stranded target nucleic acid, usually a DNA target sequence, is contacted with an SDA primer. An "SDA primer" generally has a length of 25-100 nucleotides, with SDA primers of approximately 35 nucleotides being preferred. An SDA primer is substantially complementary to a region at the 3' end of the target sequence, and the primer has a sequence at its 5' end (outside of the region that is complementary to the target) that is a recognition

sequence for a restriction endonuclease, sometimes referred to herein as a "nicking enzyme" or a "nicking endonuclease", as outlined below. The SDA primer then hybridizes to the target sequence. The SDA reaction mixture also contains a polymerase (an "SDA polymerase", as outlined below) and a mixture of all four deoxynucleoside-triphosphates (also called deoxynucleotides or dNTPs, i.e. dATP, dTTP, dCTP and dGTP), at least one species of which is a substituted or modified dNTP; thus, the SDA primer is modified, i.e. extended, to form a modified primer, sometimes referred to herein as a "newly synthesized strand". The substituted dNTP is modified such that it will inhibit cleavage in the strand containing the substituted dNTP but will not inhibit cleavage on the other strand. Examples of suitable substituted dNTPs include, but are not limited, 2'-deoxyadenosine 5'-O-(1-thiotriphosphate), 5-methyldeoxycytidine 5'-triphosphate, 2'-deoxyuridine 5'-triphosphate, and 7-deaza-2'-deoxyguanosine 5'-triphosphate. In addition, the substitution of the dNTP may occur after incorporation into a newly synthesized strand; for example, a methylase may be used to add methyl groups to the synthesized strand. In addition, if all the nucleotides are substituted, the polymerase may have 5'-3' exonuclease activity. However, if less than all the nucleotides are substituted, the polymerase preferably lacks 5'-3' exonuclease activity.

As will be appreciated by those in the art, the recognition site/endonuclease pair can be any of a wide variety of known combinations. The endonuclease is chosen to cleave a strand either at the recognition site, or either 3' or 5' to it, without cleaving the complementary sequence, either because the enzyme only cleaves one strand or because of the incorporation of the substituted nucleotides. Suitable recognition site/endonuclease pairs are well known in the art; suitable endonucleases include, but are not limited to, HincII, HindII, Aval, Fnu4HI, TthIII, NciI, BstXI, BamI, etc. A chart depicting suitable enzymes, and their corresponding recognition sites and the modified dNTP to use is found in U.S. Patent No. 5,455,166, hereby expressly incorporated by reference.

Once nicked, a polymerase (an "SDA polymerase") is used to extend the newly nicked strand, 5'-3', thereby creating another newly synthesized strand. The polymerase chosen should be able to initiate 5'-3' polymerization at a nick site, should also displace the polymerized strand downstream from the nick, and should lack 5'-3' exonuclease activity (this may be additionally accomplished by the addition of a blocking agent). Thus, suitable polymerases in SDA include, but are not limited to, the Klenow fragment of DNA polymerase I, SEQUENASE 1.0 and SEQUENASE 2.0 (U S. Biochemical), T5 DNA polymerase and Phi29 DNA polymerase.

Accordingly, the SDA reaction requires, in no particular order, an SDA primer, an SDA polymerase, a nicking endonuclease, and dNTPs, at least one species of which is modified.

In general, SDA does not require thermocycling. The temperature of the reaction is generally set to be high enough to prevent non-specific hybridization but low enough to allow specific hybridization; this is generally from about 37°C to about 42°C, depending on the enzymes.

5 In a preferred embodiment, as for most of the amplification techniques described herein, a second amplification reaction can be done using the complementary target sequence, resulting in a substantial increase in amplification during a set period of time. That is, a second primer nucleic acid is hybridized to a second target sequence, that is substantially complementary to the first target sequence, to form a second hybridization complex. The addition of the enzyme, followed by disassociation of the second hybridization complex, results in the generation of a number of newly synthesized second strands.

10 In this way, a number of target molecules are made. As is more fully outlined below, these reactions (that is, the products of these reactions) can be detected in a number of ways. In general, either direct or indirect detection of the target products can be done. "Direct" detection as used in this context, as for the other amplification strategies outlined herein, requires the incorporation of a label, in this case
15 an electron transfer moiety (ETM), into the target sequence, with detection proceeding according to either "mechanism-1" or "mechanism-2", outlined below. In this embodiment, the ETM(s) may be incorporated in three ways: (1) the primers comprise the ETM(s), for example attached to the base, a ribose, a phosphate, or to analogous structures in a nucleic acid analog; (2) modified nucleosides are used that are modified at either the base or the ribose (or to analogous structures in a nucleic acid
20 analog) with the ETM(s); these ETM modified nucleosides are then converted to the triphosphate form and are incorporated into the newly synthesized strand by a polymerase; or (3) a "tail" of ETMs can be added, as outlined below. Either of these methods result in a newly synthesized strand that comprises ETMs, that can be directly detected as outlined below.

25 Alternatively, indirect detection proceeds as a sandwich assay, with the newly synthesized strands containing few or no ETMs. Detection then proceeds via the use of label probes that comprise the ETM(s); these label probes will hybridize either directly to the newly synthesized strand or to intermediate probes such as amplification probes, as is more fully outlined below. In this case, it is the ETMs on the label probes that are used for detection as outlined below.

30 In a preferred embodiment, the target amplification technique is nucleic acid sequence based amplification (NASBA). NASBA is generally described in U.S. Patent No. 5,409,818 and "Profiting from Gene-based Diagnostics", CTB International Publishing Inc., N.J., 1996, both of which are expressly incorporated by reference in their entirety.

In general, NASBA may be described as follows. A single stranded target nucleic acid, usually an RNA target sequence (sometimes referred to herein as "the first target sequence" or "the first template"), is contacted with a first NASBA primer. A "NASBA primer" generally has a length of 25-100 nucleotides, with NASBA primers of approximately 50-75 nucleotides being preferred. The first NASBA primer is preferably a DNA primer that has at its 3' end a sequence that is substantially complementary to the 3' end of the first template. The first NASBA primer has an RNA polymerase promoter at its 5' end. The first NASBA primer is then hybridized to the first template to form a first hybridization complex. The NASBA reaction mixture also includes a reverse transcriptase enzyme (an "NASBA reverse transcriptase") and a mixture of the four dNTPs, such that the first NASBA primer is modified, i.e. extended, to form a modified first primer, comprising a hybridization complex of RNA (the first template) and DNA (the newly synthesized strand).

By "reverse transcriptase" or "RNA-directed DNA polymerase" herein is meant an enzyme capable of synthesizing DNA from a DNA primer and an RNA template. Suitable RNA-directed DNA polymerases include, but are not limited to, avian myeloblastosis virus reverse transcriptase ("AMV RT") and the Moloney murine leukemia virus RT.

In addition to the components listed above, the NASBA reaction also includes an RNA degrading enzyme, also sometimes referred to herein as a ribonuclease, that will hydrolyze RNA of an RNA:DNA hybrid without hydrolyzing single- or double-stranded RNA or DNA. Suitable ribonucleases include, but are not limited to, RNase H from *E. coli* and calf thymus.

The ribonuclease degrades the first RNA template in the hybridization complex, resulting in a disassociation of the hybridization complex leaving a first single stranded newly synthesized DNA strand, sometimes referred to herein as "the second template".

In addition, the NASBA reaction also includes a second NASBA primer, generally comprising DNA (although as for all the probes herein, including primers, nucleic acid analogs may also be used). This second NASBA primer has a sequence at its 3' end that is substantially complementary to the 3' end of the second template, and also contains an antisense sequence for a functional promoter and the antisense sequence of a transcription initiation site. Thus, this primer sequence, when used as a template for synthesis of the third DNA template, contains sufficient information to allow specific and efficient binding of an RNA polymerase and initiation of transcription at the desired site. Preferred embodiments utilizes the antisense promoter and transcription initiation site are that of the T7 RNA polymerase, although other RNA polymerase promoters and initiation sites can be used as well, as outlined below.

The second primer hybridizes to the second template, and a DNA polymerase, also termed a "DNA-directed DNA polymerase", also present in the reaction, synthesizes a third template (a second newly synthesized DNA strand), resulting in second hybridization complex comprising two newly synthesized DNA strands.

- 5 Finally, the inclusion of an RNA polymerase and the required four ribonucleoside triphosphates (ribonucleotides or NTPs) results in the synthesis of an RNA strand (a third newly synthesized strand that is essentially the same as the first template). The RNA polymerase, sometimes referred to herein as a "DNA-directed RNA polymerase", recognizes the promoter and specifically initiates RNA
10 synthesis at the initiation site. In addition, the RNA polymerase preferably synthesizes several copies of RNA per DNA duplex. Preferred RNA polymerases include, but are not limited to, T7 RNA polymerase, and other bacteriophage RNA polymerases including those of phage T3, phage ϕ II, Salmonella phage sp6, or Pseudomonase phage gh-1.

- Accordingly, the NASBA reaction requires, in no particular order, a first NASBA primer, a second NASBA primer comprising an antisense sequence of an RNA polymerase promoter, an RNA
15 polymerase that recognizes the promoter, a reverse transcriptase, a DNA polymerase, an RNA degrading enzyme, NTPs and dNTPs, in addition to the detection components outlined below.

These components result in a single starting RNA template generating a single DNA duplex; however, since this DNA duplex results in the creation of multiple RNA strands, which can then be used to initiate the reaction again, amplification proceeds rapidly.

- 20 As outlined herein, the detection of the newly synthesized strands can proceed in several ways. Direct detection can be done when the newly synthesized strands comprise ETM labels, either by incorporation into the primers or by incorporation of modified labelled nucleotides into the growing strand. Alternatively, as is more fully outlined below, indirect detection of unlabelled strands (which now serve as "targets" in the detection mode) can occur using a variety of sandwich assay
25 configurations. As will be appreciated by those in the art, it is preferable to detect DNA strands during NASBA since the presence of the ribonuclease makes the RNA strands potentially labile.

- In a preferred embodiment, the amplification technique is signal amplification. Signal amplification involves the use of limited number of target molecules as templates to either generate multiple signalling probes or allow the use of multiple signalling probes. Signal amplification strategies include
30 LCR, CPT, and the use of amplification probes in sandwich assays.

In a preferred embodiment, the signal amplification technique is LCR, as is generally depicted in Figures 21, 28 and 29. The method can be run in two different ways; in a first embodiment, only one strand of a target sequence is used as a template for ligation (Figure 28); alternatively, both strands may be used (Figure 29). See generally U.S. Patent Nos. 5,185,243 and 5,573,907; EP 0 320 308 B1; 5 EP 0 336 731 B1; EP 0 439 182 B1; WO 90/01069; WO 89/12696; and WO 89/09835, and U.S.S.N.s 60/078,102 and 60/073,011, all of which are incorporated by reference.

10 In a preferred embodiment, the single-stranded target sequence comprises a first target domain and a second target domain, and a first LCR primer and a second LCR primer nucleic acids are added, that are substantially complementary to its respective target domain and thus will hybridize to the target domains. These target domains may be directly adjacent, i.e. contiguous, or separated by a number of nucleotides. If they are non-contiguous, nucleotides are added along with means to join nucleotides, such as a polymerase, that will add the nucleotides to one of the primers. The two LCR primers are then covalently attached, for example using a ligase enzyme such as is known in the art. This forms a first hybridization complex comprising the ligated probe and the target sequence. 15 hybridization complex is then denatured (disassociated), and the process is repeated to generate a pool of ligated probes. In addition, it may be desirable to have the detection probes, described below, comprise a mismatch at the probe junction site, such that the detection probe cannot be used as a template for ligation.

20 In a preferred embodiment, LCR is done for two strands of a double-stranded target sequence. The target sequence is denatured, and two sets of probes are added: one set as outlined above for one strand of the target, and a separate set (i.e. third and fourth primer probe nucleic acids) for the other strand of the target. In a preferred embodiment, the first and third probes will hybridize, and the second and fourth probes will hybridize, such that amplification can occur. That is, when the first and second probes have been attached, the ligated probe can now be used as a template, in addition to 25 the second target sequence, for the attachment of the third and fourth probes. Similarly, the ligated third and fourth probes will serve as a template for the attachment of the first and second probes, in addition to the first target strand. In this way, an exponential, rather than just a linear, amplification can occur.

30 Again, as outlined above, the detection of the LCR reaction can occur directly, in the case where one or both of the primers comprises at least one ETM, or indirectly, using sandwich assays, through the use of additional probes; that is, the ligated probes can serve as target sequences, and detection may utilize amplification probes, capture probes, capture extender probes, label probes, and label extender probes, etc.

A variation of LCR utilizes a "chemical ligation" of sorts, as is generally outlined in U.S. Patent Nos. 5,616,464 and 5,767,259, both of which are hereby expressly incorporated by reference in their entirety. In this embodiment, similar to LCR, a pair of primers are utilized, wherein the first primer is substantially complementary to a first domain of the target and the second primer is substantially
5 complementary to an adjacent second domain of the target (although, as for LCR, if a "gap" exists, a polymerase and dNTPs may be added to "fill in" the gap). Each primer has a portion that acts as a "side chain" that does not bind the target sequence and acts one half of a stem structure that interacts non-covalently through hydrogen bonding, salt bridges, van der Waal's forces, etc. Preferred
10 embodiments utilize substantially complementary nucleic acids as the side chains. Thus, upon hybridization of the primers to the target sequence, the side chains of the primers are brought into spatial proximity, and, if the side chains comprise nucleic acids as well, can also form side chain hybridization complexes.

At least one of the side chains of the primers comprises an activatable cross-linking agent, generally covalently attached to the side chain, that upon activation, results in a chemical cross-link or chemical
15 ligation. The activatable group may comprise any moiety that will allow cross-linking of the side chains, and include groups activated chemically, photonically and thermally, with photoactivatable groups being preferred. In some embodiments a single activatable group on one of the side chains is enough to result in cross-linking via interaction to a functional group on the other side chain; in alternate
20 embodiments, activatable groups are required on each side chain.

Once the hybridization complex is formed, and the cross-linking agent has been activated such that the primers have been covalently attached, the reaction is subjected to conditions to allow for the disassociation of the hybridization complex, thus freeing up the target to serve as a template for the next ligation or cross-linking. In this way, signal amplification occurs, and can be detected as outlined
25 herein.

In a preferred embodiment, the signal amplification technique is CPT. CPT technology is described in a number of patents and patent applications, including U.S. Patent Nos. 5,011,769, 5,403,711, 5,660,988, and 4,876,187, and PCT published applications WO 95/05480, WO 95/1416, and WO 95/00667, and U.S.S.N. 09/014,304, all of which are expressly incorporated by reference in their
30 entirety.

Generally, CPT may be described as follows. A CPT primer (also sometimes referred to herein as a "scissile primer"), comprises two probe sequences separated by a scissile linkage. The CPT primer is substantially complementary to the target sequence and thus will hybridize to it to form a hybridization complex. The scissile linkage is cleaved, without cleaving the target sequence, resulting in the two

probe sequences being separated. The two probe sequences can thus be more easily disassociated from the target, and the reaction can be repeated any number of times. The cleaved primer is then detected as outlined herein.

5 By "scissile linkage" herein is meant a linkage within the scissile probe that can be cleaved when the probe is part of a hybridization complex, that is, when a double-stranded complex is formed. It is important that the scissile linkage cleave only the scissile probe and not the sequence to which it is hybridized (i.e. either the target sequence or a probe sequence), such that the target sequence may be reused in the reaction for amplification of the signal. As used herein, the scissile linkage, is any connecting chemical structure which joins two probe sequences and which is capable of being
10 selectively cleaved without cleavage of either the probe sequences or the sequence to which the scissile probe is hybridized. The scissile linkage may be a single bond, or a multiple unit sequence. As will be appreciated by those in the art, a number of possible scissile linkages may be used.

In a preferred embodiment, the scissile linkage comprises RNA. This system, previously described in as outlined above, is based on the fact that certain double-stranded nucleases, particularly
15 ribonucleases, will nick or excise RNA nucleosides from a RNA:DNA hybridization complex. Of particular use in this embodiment is RNaseH, Exo III, and reverse transcriptase.

In one embodiment, the entire scissile probe is made of RNA, the nicking is facilitated especially when carried out with a double-stranded ribonuclease, such as RNaseH or Exo III. RNA probes made entirely of RNA sequences are particularly useful because first, they can be more easily produced
20 enzymatically, and second, they have more cleavage sites which are accessible to nicking or cleaving by a nicking agent, such as the ribonucleases. Thus, scissile probes made entirely of RNA do not rely on a scissile linkage since the scissile linkage is inherent in the probe.

In a preferred embodiment, when the scissile linkage is a nucleic acid such as RNA, the methods of the invention may be used to detect mismatches, as is generally described in U.S. Patent Nos.
25 5,660,988, and WO 95/14106, hereby expressly incorporated by reference. These mismatch detection methods are based on the fact that RNaseH may not bind to and/or cleave an RNA:DNA duplex if there are mismatches present in the sequence. Thus, in the NA_1 -R- NA_2 embodiments, NA_1 and NA_2 are non-RNA nucleic acids, preferably DNA. Preferably, the mismatch is within the RNA:DNA duplex, but in some embodiments the mismatch is present in an adjacent sequence very close to the
30 desired sequence, close enough to affect the RNaseH (generally within one or two bases). Thus, in this embodiment, the nucleic acid scissile linkage is designed such that the sequence of the scissile linkage reflects the particular sequence to be detected, i.e. the area of the putative mismatch.

In some embodiments of mismatch detection, the rate of generation of the released fragments is such that the methods provide, essentially, a yes/no result, whereby the detection of the virtually any released fragment indicates the presence of the desired target sequence. Typically, however, when there is only a minimal mismatch (for example, a 1-, 2- or 3-base mismatch, or a 3-base deletion), there is some generation of cleaved sequences even though the target sequence is not present. Thus, the rate of generation of cleaved fragments, and/or the final amount of cleaved fragments, is quantified to indicate the presence or absence of the target. In addition, the use of secondary and tertiary scissile probes may be particularly useful in this embodiment, as this can amplify the differences between a perfect match and a mismatch. These methods may be particularly useful in the determination of homozygotic or heterozygotic states of a patient.

In this embodiment, it is an important feature of the scissile linkage that its length is determined by the suspected difference between the target and the probe. In particular, this means that the scissile linkage must be of sufficient length to encompass the suspected difference, yet short enough the scissile linkage cannot inappropriately "specifically hybridize" to the selected nucleic acid molecule when the suspected difference is present; such inappropriate hybridization would permit excision and thus cleavage of scissile linkages even though the selected nucleic acid molecule was not fully complementary to the nucleic acid probe. Thus in a preferred embodiment, the scissile linkage is between 3 to 5 nucleotides in length, such that a suspected nucleotide difference from 1 nucleotide to 3 nucleotides is encompassed by the scissile linkage, and 0, 1 or 2 nucleotides are on either side of the difference.

Thus, when the scissile linkage is nucleic acid, preferred embodiments utilize from 1 to about 100 nucleotides, with from about 2 to about 20 being preferred and from about 5 to about 10 being particularly preferred.

CPT may be done enzymatically or chemically. That is, in addition to RNaseH, there are several other cleaving agents which may be useful in cleaving RNA (or other nucleic acid) scissile bonds. For example, several chemical nucleases have been reported; see for example Sigman et al., *Annu. Rev. Biochem.* 1990, 59, 207-236; Sigman et al., *Chem. Rev.* 1993, 93, 2295-2316; Bashkin et al., *J. Org. Chem.* 1990, 55, 5125-5132; and Sigman et al., *Nucleic Acids and Molecular Biology*, vol. 3, F. Eckstein and D.M.J. Lilley (Eds), Springer-Verlag, Heidelberg 1989, pp. 13-27; all of which are hereby expressly incorporated by reference.

Specific RNA hydrolysis is also an active area; see for example Chin, *Acc. Chem. Res.* 1991, 24, 145-152; Breslow et al., *Tetrahedron*, 1991, 47, 2365-2376; Anslyn et al., *Angew. Chem. Int. Ed. Engl.*, 1997, 36, 432-450; and references therein, all of which are expressly incorporated by reference.

Reactive phosphate centers are also of interest in developing scissile linkages, see Hendry et al., Prog. Inorg. Chem. : Bioinorganic Chem. 1990, 31, 201-258 also expressly incorporated by reference.

Current approaches to site-directed RNA hydrolysis include the conjugation of a reactive moiety capable of cleaving phosphodiester bonds to a recognition element capable of sequence-specifically hybridizing to RNA. In most cases, a metal complex is covalently attached to a DNA strand which forms a stable heteroduplex. Upon hybridization, a Lewis acid is placed in close proximity to the RNA backbone to effect hydrolysis; see Magda et al., J. Am. Chem. Soc. 1994, 116, 7439; Hall et al., Chem. Biology 1994, 1, 185-190; Bashkin et al., J. Am. Chem. Soc. 1994, 116, 5981-5982; Hall et al., Nucleic Acids Res. 1996, 24, 3522; Magda et al., J. Am. Chem. Soc. 1997, 119, 2293; and Magda et al., J. Am. Chem. Soc. 1997, 119, 6947, all of which are expressly incorporated by reference.

In a similar fashion, DNA-polyamine conjugates have been demonstrated to induce site-directed RNA strand scission; see for example, Yoshinari et al., J. Am. Chem. Soc. 1991, 113, 5899-5901; Endo et al., J. Org. Chem. 1997, 62, 846; and Barbier et al., J. Am. Chem. Soc. 1992, 114, 3511-3515, all of which are expressly incorporated by reference.

In a preferred embodiment, the scissile linkage is not necessarily RNA. For example, chemical cleavage moieties may be used to cleave basic sites in nucleic acids; see Belmont, et al., New J. Chem. 1997, 21, 47-54; and references therein, all of which are expressly incorporated herein by reference. Similarly, photocleavable moieties, for example, using transition metals, may be used; see Moucheron, et al., Inorg. Chem. 1997, 36, 584-592, hereby expressly by reference.

Other approaches rely on chemical moieties or enzymes; see for example Keck et al., Biochemistry 1995, 34, 12029-12037; Kirk et al., Chem. Commun. 1998, in press; cleavage of G-U basepairs by metal complexes; see Biochemistry, 1992, 31, 5423-5429; diamine complexes for cleavage of RNA; Komiyama, et al., J. Org. Chem. 1997, 62, 2155-2160; and Chow et al., Chem. Rev. 1997, 97, 1489-1513, and references therein, all of which are expressly incorporated herein by reference.

The first step of the CPT method requires hybridizing a primary scissile primer (also called a primary scissile probe) to the target. This is preferably done at a temperature that allows both the binding of the longer primary probe and disassociation of the shorter cleaved portions of the primary probe, as will be appreciated by those in the art. As outlined herein, this may be done in solution, or either the target or one or more of the scissile probes may be attached to a solid support. For example, it is possible to utilize "anchor probes" on a solid support or the electrode which are substantially complementary to a portion of the target sequence, preferably a sequence that is not the same sequence to which a scissile probe will bind.

Similarly, as outlined herein, a preferred embodiment has one or more of the scissile probes attached to a solid support such as a bead. In this embodiment, the soluble target diffuses to allow the formation of the hybridization complex between the soluble target sequence and the support-bound scissile probe. In this embodiment, it may be desirable to include additional scissile linkages in the scissile probes to allow the release of two or more probe sequences, such that more than one probe sequence per scissile probe may be detected, as is outlined below, in the interests of maximizing the signal. Such embodiments are generally depicted in Figures 34 and 35.

In this embodiment (and in other amplification techniques herein), preferred methods utilize cutting or shearing techniques to cut the nucleic acid sample containing the target sequence into a size that will allow sufficient diffusion of the target sequence to the surface of a bead. This may be accomplished by shearing the nucleic acid through mechanical forces (e.g. sonication) or by cleaving the nucleic acid using restriction endonucleases. Alternatively, a fragment containing the target may be generated using polymerase, primers and the sample as a template, as in polymerase chain reaction (PCR). In addition, amplification of the target using PCR or LCR or related methods may also be done; this may be particularly useful when the target sequence is present in the sample at extremely low copy numbers. Similarly, numerous techniques are known in the art to increase the rate of mixing and hybridization including agitation, heating, techniques that increase the overall concentration such as precipitation, drying, dialysis, centrifugation, electrophoresis, magnetic bead concentration, etc.

In general, the scissile probes are introduced in a molar excess to their targets (including both the target sequence or other scissile probes, for example when secondary or tertiary scissile probes are used), with ratios of scissile probe:target of at least about 100:1 being preferred, at least about 1000:1 being particularly preferred, and at least about 10,000:1 being especially preferred. In some embodiments the excess of probe:target will be much greater. In addition, ratios such as these may be used for all the amplification techniques outlined herein.

Once the hybridization complex between the primary scissile probe and the target has been formed, the complex is subjected to cleavage conditions. As will be appreciated, this depends on the composition of the scissile probe; if it is RNA, RNaseH is introduced. It should be noted that under certain circumstances, such as is generally outlined in WO 95/00666 and WO 95/00667, hereby incorporated by reference, the use of a double-stranded binding agent such as RNaseH may allow the reaction to proceed even at temperatures above the T_m of the primary probe:target hybridization complex. Accordingly, the addition of scissile probe to the target can be done either first, and then the cleavage agent or cleavage conditions introduced, or the probes may be added in the presence of the cleavage agent or conditions.

The cleavage conditions result in the separation of the two (or more) probe sequences of the primary scissile probe. As a result, the shorter probe sequences will no longer remain hybridized to the target sequence, and thus the hybridization complex will disassociate, leaving the target sequence intact. The optimal temperature for carrying out the CPT reactions is generally from about 5°C to about 25°C
5 below the melting temperatures of the probe:target hybridization complex. This provides for a rapid rate of hybridization and high degree of specificity for the target sequence. The T_m of any particular hybridization complex depends on salt concentration, G-C content, and length of the complex, as is known in the art.

During the reaction, as for the other amplification techniques herein, it may be necessary to suppress
10 cleavage of the probe, as well as the target sequence, by nonspecific nucleases. Such nucleases are generally removed from the sample during the isolation of the DNA by heating or extraction procedures. A number of inhibitors of single-stranded nucleases such as vanadate, inhibitors of ACE and RNaseH, a placental protein, do not affect the activity of RNaseH. This may not be necessary depending on the purity of the RNaseH and/or the target sample.

These steps are repeated by allowing the reaction to proceed for a period of time. The reaction is usually carried out for about 15 minutes to about 1 hour. Generally, each molecule of the target sequence will turnover between 100 and 1000 times in this period, depending on the length and sequence of the probe, the specific reaction conditions, and the cleavage method. For example, for
15 each copy of the target sequence present in the test sample 100 to 1000 molecules will be cleaved by
20 RNaseH. Higher levels of amplification can be obtained by allowing the reaction to proceed longer, or using secondary, tertiary, or quaternary probes, as is outlined herein.

Upon completion of the reaction, generally determined by time or amount of cleavage, the uncleaved scissile probes must be removed or neutralized prior to detection, such that the uncleaved probe does not bind to a detection probe, causing false positive signals. This may be done in a variety of ways, as
25 is generally described below.

In a preferred embodiment, the separation is facilitated by the use of beads containing the primary probe. Thus, when the scissile probes are attached to beads, removal of the beads by filtration, centrifugation, the application of a magnetic field, electrostatic interactions for charged beads, adhesion, etc., results in the removal of the uncleaved probes.

30 In a preferred embodiment, the separation is based on gel electrophoresis of the reaction products to separate the longer uncleaved probe from the shorter cleaved probe sequences as is known in the art.

In a preferred embodiment, the separation is based on strong acid precipitation. This is useful to separate long (generally greater than 50 nucleotides) from smaller fragments (generally about 10 nucleotides). The introduction of a strong acid such as trichloroacetic acid into the solution causes the longer probe to precipitate, while the smaller cleaved fragments remain in solution. The solution can be centrifuged or filtered to remove the precipitate, and the cleaved probe sequences can be quantitated.

In a preferred embodiment, the scissile probe contains both an ETM and an affinity binding ligand or moiety, such that an affinity support is used to carry out the separation. In this embodiment, it is important that the ETM used for detection is not on the same probe sequence that contains the affinity moiety, such that removal of the uncleaved probe, and the cleaved probe containing the affinity moiety, does not remove all the detectable ETMs. Alternatively, the scissile probe may not contain a covalently attached ETM, but just an affinity label. Suitable affinity moieties include, but are not limited to, biotin, avidin, streptavidin, lectins, haptens, antibodies, etc. The binding partner of the affinity moiety is attached to a solid support such as glass beads, latex beads, dextrans, etc. and used to pull out the uncleaved probes, as is known in the art. The cleaved probe sequences, which do not contain the affinity moiety, remain in solution and then can be detected as outlined below.

In a preferred embodiment, similar to the above embodiment, a separation sequence of nucleic acid is included in the scissile probe, which is not cleaved during the reaction. A nucleic acid complementary to the separation sequence is attached to a solid support such as a bead and serves as a catcher sequence. Preferably, the separation sequence is added to the scissile probes, and is not recognized by the target sequence, such that a generalized catcher sequence may be utilized in a variety of assays.

In a preferred embodiment, the uncleaved probe is neutralized by the addition of a substantially complementary neutralization nucleic acid, as is generally depicted in Figure 32. This is particularly useful in embodiments utilizing capture sequences, separation sequences, and one-step systems, as the complement to a probe containing capture sequences forms hybridization complexes that are more stable due to its length than the cleaved probe sequence:detection probe complex. As will be appreciated by those in the art, complete removal of the uncleaved probe is not required, since detection is based on electron transfer through nucleic acid; rather, what is important is that the uncleaved probe is not available for binding to a detection electrode probe specific for cleaved sequences. Thus, in one embodiment, the neutralization nucleic acid is a detection probe on the surface of the electrode, at a separate "address", such that the signal from the neutralization hybridization complex does not contribute to the signal of the cleaved fragments. Alternatively, the

neutralization nucleic acid may be attached to a bead; the neutralization beads are added to quench the reaction, and then removed prior to detection.

After removal or neutralization of the uncleaved probe, detection proceeds via the addition of the cleaved probe sequences to the detection compositions, as outlined below, which can utilize either "mechanism-1" or "mechanism-2" systems. A mechanism-1 system can be described as follows; the cleaved probe sequences hybridize to a first detection single-stranded probe covalently attached via a conductive oligomer to an electrode, that thus forms a second hybridization complex. The second hybridization complex, comprising detection probe:probe sequence, contains at least a first ETM. As outlined herein, this ETM may be covalently attached to either the probe (primer) sequence or the detection probe, or it may be added non-covalently as a hybridization indicator, or both. As outlined above, preferred embodiments utilize more than one ETM per hybridization complex for detection.

In a preferred embodiment, no higher order probes are used, and detection is based on the probe sequence(s) of the primary primer. Thus, in a preferred embodiment, the electrode comprises at least a first detection probe which is substantially complementary to all or part of a cleaved portion of the primary scissile probe. In one embodiment, only one type of detection probe is utilized, which can be substantially complementary to all or part of any probe sequence of the primary probe. In a preferred embodiment, more than one type of detection probe is utilized, with each detection probe being substantially complementary to all or part of each probe sequence of the primary probe. Thus, when the primary probe comprises two probe sequences, two detection probes are used; three probe sequences utilizes three detection probes. This may require the use of additional scissile linkages when the probes are bound to beads, as is described herein. The detection probes and the primary probe sequences then form hybridization complexes, which either contain covalently bound ETMs or ETMs in the form of hybridization indicators are added to the system (or both), which are then detected as is outlined herein. In a preferred embodiment, when hybridization indicators are used, they are only added to the system after the reaction is complete, to avoid the association of hybridization indicators to the probe:target complexes, although in some embodiments it may be possible to have the hybridization indicators present during the reaction.

In a preferred embodiment, the detection probes are mixed on the surface of the electrode, such that the signal from each is combined. This may be particularly preferred when the target sequence is present in low copy number. Alternatively, the detection probes may each be at a different "address" on the surface. While this reduces the possible signal from the system, it serves as an internal control in that the signal from each should be equal, all other parameters being equal. In addition, different addresses can have different densities of probes creating addresses with various sensitivities to target

sequences. Systems utilizing two detection probes, each to a probe sequence of the primary probe, that is bound to a bead, are generally depicted in Figure 30, utilizing bound ETMs.

5 In a preferred embodiment, at least one, and preferably more, secondary probes (also referred to herein as secondary primers) are used. The secondary scissile probes may be added to the reaction in several ways. It is important that the secondary scissile probes be prevented from hybridizing to the uncleaved primary probes, as this results in the generation of false positive signal. These methods may be described in several ways, depending on whether bead-bound probes are used.

10 In a preferred embodiment, the primary and secondary probes are bound to solid supports. In a preferred embodiment, the primary and secondary probes are added together, since generally the support-bound secondary probes will be unable to bind to the uncleaved primary probes on the surface of a bead. It is only upon hybridization of the primary probes with the target, resulting in cleavage and release of primary probe sequences from the bead, that the now diffusible primary probe sequences may bind to the secondary probes. In turn, the primary probe sequences serve as targets for the secondary scissile probes, resulting in cleavage and release of secondary probe sequences.

15 In an alternate embodiment, the beads containing the primary probes are added, the reaction is allowed to proceed for some period of time, and then the beads containing the secondary probes are added, either with removal of the primary beads or not. Alternatively, the beads containing the primary probes are removed and soluble secondary scissile probes are added.

20 In an alternate embodiment, the complete reaction is done in solution. In this embodiment, the primary probes are added, the reaction is allowed to proceed for some period of time, and the uncleaved primary scissile probes are removed, as outlined above. The secondary probes are then added, and the reaction proceeds. The secondary uncleaved probes are then removed, and the cleaved sequences are detected as is generally outlined herein.

25 As above, it is generally preferred to detect as many secondary probe sequences as possible, and the primary probe sequences may be additionally detected as well. Thus, preferred embodiments utilize detection probes that are substantially complementary to all or part of each probe sequence of a scissile probe. Alternatively, only detection probes for "higher order" probe sequences are used. Furthermore, in some embodiments, detection probes for only one of the probe sequences of any scissile probe may be used. Furthermore, as outlined above, in these embodiments, as for the
30 others, the detection probes may be separated by sequence, or mixed, depending on the desired results.

In a preferred embodiment, at least one, and preferably more, tertiary probes are used. The tertiary scissile probes may be added to the reaction in several ways. It is important that the tertiary scissile probes be prevented from hybridizing to the uncleaved secondary probes, as this results in the generation of false positive signal. These methods may be described in several ways, depending on whether bead-bound probes are used.

In a preferred embodiment, the primary, secondary and tertiary probes are bound to solid supports. In a preferred embodiment, the primary, secondary and tertiary probes are added together, since generally the support-bound secondary probes will be unable to bind to the uncleaved primary probes on the surface of a bead and the support-bound tertiary probes will be unable to bind to the uncleaved secondary probes on the surface of a bead. It is only upon hybridization of the scissile probe with its target (i.e. either the target sequences of the sample for the primary probes, or probe sequences for each of the secondary and tertiary probes), that results in cleavage and release of probe sequences from the bead, that the now diffusable probe sequences may bind to the higher order probes.

In alternate embodiments, combinations of beads and solution probes are used, with any combination being possible: primary probe beads, soluble secondary probes, tertiary beads; soluble primary probes, soluble secondary probes, tertiary beads; primary probe beads, secondary probe beads, soluble tertiary probes; etc. What is important is that if soluble probes are used, they must be removed prior to the addition of the next higher order probe.

In an alternate embodiment, the complete reaction is done in solution. In this embodiment, the primary probes are added, the reaction is allowed to proceed for some period of time, and the uncleaved primary scissile probes are removed, as outlined above. The secondary probes are then added, and the reaction proceeds. The secondary uncleaved probes are then removed, and the tertiary probes are added, and the reaction proceeds. The uncleaved tertiary probes are then removed and the cleaved sequences are detected as is generally outlined herein.

As above, it is generally preferred to detect as many tertiary probe sequences as possible, and the secondary and primary probe sequences may be additionally detected as well. Thus, preferred embodiments utilize detection probes that are substantially complementary to all or part of each probe sequence of a scissile probe. Again, the detection sequences may be separated on the electrode, or mixed, to allow the greatest signal amplification.

In a preferred embodiment, at least one, and preferably more, quaternary probes are used. This proceeds as outlined above for tertiary probes.

Thus, CPT requires, again in no particular order, a first CPT primer comprising a first probe sequence, a scissile linkage and a second probe sequence; and a cleavage agent.

In this manner, CPT results in the generation of a large amount of cleaved primers, which then can be detected as outlined below.

5 In a preferred embodiment, the signal amplification technique is a "sandwich" assay, as is generally described in U.S.S.N. 60/073,011 and in U.S. Patent Nos. 5,681,702, 5,597,909, 5,545,730, 5,594,117, 5,591,584, 5,571,670, 5,580,731, 5,571,670, 5,591,584, 5,624,802, 5,635,352, 5,594,118, 5,359,100, 5,124,246 and 5,681,697, all of which are hereby incorporated by reference. Although sandwich assays do not result in the alteration of primers, sandwich assays can be considered signal
10 amplification techniques since multiple signals (i.e. label probes) are bound to a single target, resulting in the amplification of the signal. Sandwich assays are used when the target sequence comprises little or no ETM labels; that is, when a secondary probe, comprising the ETM labels, is used to generate the signal.

As discussed herein, it should be noted that the sandwich assays can be used for the detection of
15 primary target sequences (e.g. from a patient sample), or as a method to detect the product of an amplification reaction as outlined above; thus for example, any of the newly synthesized strands outlined above, for example using PCR, LCR, NASBA, SDA, etc., may be used as the "target sequence" in a sandwich assay.

Generally, sandwich signal amplification techniques may be described as follows. In preferred
20 embodiments, although it is not required, the target sequences are immobilized on the electrode surface. This is preferably done using capture probes and optionally one or more capture extender probes; see Figure 15. When only capture probes are utilized, it is necessary to have unique capture probes for each target sequence; that is, the surface must be customized to contain unique capture probes. Alternatively, capture extender probes may be used, that allow a "universal" surface, i.e. a
25 surface containing a single type of capture probe that can be used to detect any target sequence. "Capture extender" probes are generally depicted in Figure 15, and other figures, and have a first portion that will hybridize to all or part of the capture probe, and a second portion that will hybridize to a first portion of the target sequence. This then allows the generation of customized soluble probes, which as will be appreciated by those in the art is generally simpler and less costly. As shown herein,
30 two capture extender probes may be used. This has generally been done to stabilize assay complexes (for example when the target sequence is large, or when large amplifier probes (particularly branched or dendrimer amplifier probes) are used.

In a preferred embodiment, the nucleic acids are added after the formation of the SAM, discussed below. This may be done in a variety of ways, as will be appreciated by those in the art. In one embodiment, conductive oligomers with terminal functional groups are made, with preferred embodiments utilizing activated carboxylates and isothiocyanates, that will react with primary amines that are put onto the nucleic acid, as is generally depicted in Figure 6 using an activated carboxylate. These two reagents have the advantage of being stable in aqueous solution, yet react with primary alkylamines. However, the primary aromatic amines and secondary and tertiary amines of the bases should not react, thus allowing site specific addition of nucleic acids to the surface. This allows the spotting of probes (either capture or detection probes, or both) using known methods (ink jet, spotting, etc.) onto the surface.

In addition, there are a number of non-nucleic acid methods that can be used to immobilize a nucleic acid on a surface. For example, binding partner pairs can be utilized; i.e. one binding partner is attached to the terminus of an attachment linker, described below, and the other to the end of the nucleic acid. This may also be done without using a nucleic acid capture probe; that is, one binding partner serves as the capture probe and the other is attached to either the target sequence or a capture extender probe. That is, either the target sequence comprises the binding partner, or a capture extender probe that will hybridize to the target sequence comprises the binding partner. Suitable binding partner pairs include, but are not limited to, hapten pairs such as biotin/streptavidin; antigens/antibodies; NTA/histidine tags; etc. In general, smaller binding partners are preferred, such that the electrons can pass from the nucleic acid into the conductive oligomer to allow detection.

In a preferred embodiment, when the target sequence itself is modified to contain a binding partner, the binding partner is attached via a modified nucleotide that can be enzymatically attached to the target sequence, for example during a PCR target amplification step. Alternatively, the binding partner should be easily attached to the target sequence.

Alternatively, a capture extender probe may be utilized that has a nucleic acid portion for hybridization to the target as well as a binding partner (for example, the capture extender probe may comprise a non-nucleic acid portion such as an alkyl linker that is used to attach a binding partner). In this embodiment, it may be desirable to cross-link the double-stranded nucleic acid of the target and capture extender probe for stability, for example using psoralen as is known in the art.

In one embodiment, the target is not bound to the electrode surface using capture probes. In this embodiment, what is important, as for all the assays herein, is that excess label probes be removed prior to detection and that the assay complex be in proximity to the surface. As will be appreciated by those in the art, this may be accomplished in other ways. For example, the assay complex comprising

the ETMs may be present on beads that are added to the electrode comprising the monolayer, and then the beads brought into proximity of the electrode surface using techniques well known in the art, including gravity settling of the beads on the surface, electrostatic or magnetic interactions between bead components and the surface, using binding partner attachment as outlined above. Alternatively, after the removal of excess reagents such as excess label probes, the assay complex may be driven down to the surface, for example by pulsing the system with a voltage sufficient to drive the assay complex to the surface.

However, preferred embodiments utilize assay complexes attached via nucleic acid capture probes.

Once the target sequence has preferably been anchored to the electrode, an amplifier probe is hybridized to the target sequence, either directly, or through the use of one or more label extender probes, which serves to allow "generic" amplifier probes to be made. Preferably, the amplifier probe contains a multiplicity of amplification sequences, although in some embodiments, as described below, the amplifier probe may contain only a single amplification sequence, or at least two amplification sequences. The amplifier probe may take on a number of different forms; either a branched conformation, a dendrimer conformation, or a linear "string" of amplification sequences. Label probes comprising ETMs then hybridize to the amplification sequences (or in some cases the label probes hybridize directly to the target sequence), and the ETMs are detected using the electrode, as is more fully outlined below.

As will be appreciated by those in the art, the systems of the invention may take on a large number of different configurations, as is generally depicted in Figures 15, 16, 27, etc. In general, there are three types of systems that can be used: (1) "non-sandwich" systems (also referred to herein as "direct" detection) in which the target sequence itself is labeled with ETMs (again, either because the primers comprise ETMs or due to the incorporation of ETMs into the newly synthesized strand); (2) systems in which label probes directly bind to the target analytes; and (3) systems in which label probes are indirectly bound to the target sequences, for example through the use of amplifier probes.

Accordingly, the present invention provides compositions comprising an amplifier probe. By "amplifier probe" or "nucleic acid multimer" or "amplification multimer" or grammatical equivalents herein is meant a nucleic acid probe that is used to facilitate signal amplification. Amplifier probes comprise at least a first single-stranded nucleic acid probe sequence, as defined below, and at least one single-stranded nucleic acid amplification sequence, with a multiplicity of amplification sequences being preferred.

Amplifier probes comprise a first probe sequence that is used, either directly or indirectly, to hybridize to the target sequence. That is, the amplifier probe itself may have a first probe sequence that is substantially complementary to the target sequence, or it has a first probe sequence that is substantially complementary to a portion of an additional probe, in this case called a label extender probe, that has a first portion that is substantially complementary to the target sequence. In a preferred embodiment, the first probe sequence of the amplifier probe is substantially complementary to the target sequence.

In general, as for all the probes herein, the first probe sequence is of a length sufficient to give specificity and stability. Thus generally, the probe sequences of the invention that are designed to hybridize to another nucleic acid (i.e. probe sequences, amplification sequences, portions or domains of larger probes) are at least about 5 nucleosides long, with at least about 10 being preferred and at least about 15 being especially preferred.

In a preferred embodiment, as is depicted in Figure 18, the amplifier probes, or any of the other probes of the invention, may form hairpin stem-loop structures in the absence of their target. The length of the stem double-stranded sequence will be selected such that the hairpin structure is not favored in the presence of target. The use of these type of probes, in the systems of the invention or in any nucleic acid detection systems, can result in a significant decrease in non-specific binding and thus an increase in the signal to noise ratio.

Generally, these hairpin structures comprise four components. The first component is a target binding sequence, i.e. a region complementary to the target (which may be the sample target sequence or another probe sequence to which binding is desired), that is about 10 nucleosides long, with about 15 being preferred. The second component is a loop sequence, that can facilitate the formation of nucleic acid loops. Particularly preferred in this regard are repeats of GTC, which has been identified in Fragile X Syndrome as forming turns. (When PNA analogs are used, turns comprising proline residues may be preferred). Generally, from three to five repeats are used, with four to five being preferred. The third component is a self-complementary region, which has a first portion that is complementary to a portion of the target sequence region and a second portion that comprises a first portion of the label probe binding sequence. The fourth component is substantially complementary to a label probe (or other probe, as the case may be). The fourth component further comprises a "sticky end", that is, a portion that does not hybridize to any other portion of the probe, and preferably contains most, if not all, of the ETMs. The general structure is depicted in Figure 18. As will be appreciated by those in the art, the any or all of the probes described herein may be configured to form hairpins in the absence of their targets, including the amplifier, capture, capture extender, label and label extender probes.

In a preferred embodiment, several different amplifier probes are used, each with first probe sequences that will hybridize to a different portion of the target sequence. That is, there is more than one level of amplification; the amplifier probe provides an amplification of signal due to a multiplicity of labelling events, and several different amplifier probes, each with this multiplicity of labels, for each target sequence is used. Thus, preferred embodiments utilize at least two different pools of amplifier probes, each pool having a different probe sequence for hybridization to different portions of the target sequence; the only real limitation on the number of different amplifier probes will be the length of the original target sequence. In addition, it is also possible that the different amplifier probes contain different amplification sequences, although this is generally not preferred.

In a preferred embodiment, the amplifier probe does not hybridize to the sample target sequence directly, but instead hybridizes to a first portion of a label extender probe. This is particularly useful to allow the use of "generic" amplifier probes, that is, amplifier probes that can be used with a variety of different targets. This may be desirable since several of the amplifier probes require special synthesis techniques. Thus, the addition of a relatively short probe as a label extender probe is preferred. Thus, the first probe sequence of the amplifier probe is substantially complementary to a first portion or domain of a first label extender single-stranded nucleic acid probe. The label extender probe also contains a second portion or domain that is substantially complementary to a portion of the target sequence. Both of these portions are preferably at least about 10 to about 50 nucleotides in length, with a range of about 15 to about 30 being preferred. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target or probe sequences. For example, assuming a 5'-3' orientation of the complementary target sequence, the first portion may be located either 5' to the second portion, or 3' to the second portion. For convenience herein, the order of probe sequences are generally shown from left to right.

In a preferred embodiment, more than one label extender probe-amplifier probe pair may be used, that is, n is more than 1. That is, a plurality of label extender probes may be used, each with a portion that is substantially complementary to a different portion of the target sequence; this can serve as another level of amplification. Thus, a preferred embodiment utilizes pools of at least two label extender probes, with the upper limit being set by the length of the target sequence.

In a preferred embodiment, more than one label extender probe is used with a single amplifier probe to reduce non-specific binding, as is generally outlined in U.S. Patent No. 5,681,697, incorporated by reference herein. In this embodiment, a first portion of the first label extender probe hybridizes to a first portion of the target sequence, and the second portion of the first label extender probe hybridizes to a first probe sequence of the amplifier probe. A first portion of the second label extender probe hybridizes to a second portion of the target sequence, and the second portion of the second label

extender probe hybridizes to a second probe sequence of the amplifier probe. These form structures sometimes referred to as "cruciform" structures or configurations, and are generally done to confer stability when large branched or dendrimeric amplifier probes are used.

5 In addition, as will be appreciated by those in the art, the label extender probes may interact with a preamplifier probe, described below, rather than the amplifier probe directly.

Similarly, as outlined above, a preferred embodiment utilizes several different amplifier probes, each with first probe sequences that will hybridize to a different portion of the label extender probe. In addition, as outlined above, it is also possible that the different amplifier probes contain different amplification sequences, although this is generally not preferred.

10 In addition to the first probe sequence, the amplifier probe also comprises at least one amplification sequence. An "amplification sequence" or "amplification segment" or grammatical equivalents herein is meant a sequence that is used, either directly or indirectly, to bind to a first portion of a label probe as is more fully described below (although in some cases the amplification sequence may bind to a detection probe; see Figure 27C). Preferably, the amplifier probe comprises a multiplicity of
15 amplification sequences, with from about 3 to about 1000 being preferred, from about 10 to about 100 being particularly preferred, and about 50 being especially preferred. In some cases, for example when linear amplifier probes are used, from 1 to about 20 is preferred with from about 5 to about 10 being particularly preferred.

20 The amplification sequences may be linked to each other in a variety of ways, as will be appreciated by those in the art. They may be covalently linked directly to each other, or to intervening sequences or chemical moieties, through nucleic acid linkages such as phosphodiester bonds, PNA bonds, etc., or through interposed linking agents such amino acid, carbohydrate or polyol bridges, or through other cross-linking agents or binding partners. The site(s) of linkage may be at the ends of a segment, and/or at one or more internal nucleotides in the strand. In a preferred embodiment, the amplification
25 sequences are attached via nucleic acid linkages.

In a preferred embodiment, branched amplifier probes are used, as are generally described in U.S. Patent No. 5,124,246, hereby incorporated by reference. Branched amplifier probes may take on "fork-like" or "comb-like" conformations. "Fork-like" branched amplifier probes generally have three or more oligonucleotide segments emanating from a point of origin to form a branched structure. The
30 point of origin may be another nucleotide segment or a multifunctional molecule to which at least three segments can be covalently or tightly bound. "Comb-like" branched amplifier probes have a linear backbone with a multiplicity of sidechain oligonucleotides extending from the backbone. In either

conformation, the pendant segments will normally depend from a modified nucleotide or other organic moiety having the appropriate functional groups for attachment of oligonucleotides. Furthermore, in either conformation, a large number of amplification sequences are available for binding, either directly or indirectly, to detection probes. In general, these structures are made as is known in the art, using modified multifunctional nucleotides, as is described in U.S. Patent Nos. 5,635,352 and 5,124,246, among others.

In a preferred embodiment, dendrimer amplifier probes are used, as are generally described in U.S. Patent No. 5,175,270, hereby expressly incorporated by reference. Dendrimeric amplifier probes have amplification sequences that are attached via hybridization, and thus have portions of double-stranded nucleic acid as a component of their structure. The outer surface of the dendrimer amplifier probe has a multiplicity of amplification sequences.

In a preferred embodiment, linear amplifier probes are used, that have individual amplification sequences linked end-to-end either directly or with short intervening sequences to form a polymer. As with the other amplifier configurations, there may be additional sequences or moieties between the amplification sequences. In addition, as outlined herein, linear amplification probes may form hairpin stem-loop structures, as is depicted in Figure 18.

In one embodiment, the linear amplifier probe has a single amplification sequence. This may be useful when cycles of hybridization/disassociation occurs, forming a pool of amplifier probe that was hybridized to the target and then removed to allow more probes to bind, or when large numbers of ETMs are used for each label probe. However, in a preferred embodiment, linear amplifier probes comprise a multiplicity of amplification sequences.

In addition, the amplifier probe may be totally linear, totally branched, totally dendrimeric, or any combination thereof.

The amplification sequences of the amplifier probe are used, either directly or indirectly, to bind to a label probe to allow detection. In a preferred embodiment, the amplification sequences of the amplifier probe are substantially complementary to a first portion of a label probe. Alternatively, amplifier extender probes are used, that have a first portion that binds to the amplification sequence and a second portion that binds to the first portion of the label probe.

In addition, the compositions of the invention may include "preamplifier" molecules, which serves a bridging moiety between the label extender molecules and the amplifier probes. In this way, more

amplifier and thus more ETMs are ultimately bound to the detection probes. Preamplifier molecules may be either linear or branched, and typically contain in the range of about 30-3000 nucleotides.

Thus, label probes are either substantially complementary to an amplification sequence or to a portion of the target sequence. Accordingly, the label probes can be configured in a variety of ways, as is generally described herein, depending on whether a "mechanism-1" or "mechanism-2" detection system is utilized, as described below.

Detection of the amplification reactions of the invention, including the direct detection of amplification products and indirect detection utilizing label probes (i.e. sandwich assays), is done by detecting assay complexes comprising ETMs, which can be attached to the assay complex in a variety of ways, as is more fully described below. In general, there are two basic detection mechanisms. In a preferred embodiment, detection of an ETM is based on electron transfer through the stacked π -orbitals of double stranded nucleic acid. This basic mechanism is described in U.S. Patent Nos. 5,591,578, 5,770,369, 5,705,348, 5,824,473 and 5,780,234 and WO98/20162, all of which are expressly incorporated by reference, and is termed "mechanism-1" herein. Briefly, previous work has shown that electron transfer can proceed rapidly through the stacked π -orbitals of double stranded nucleic acid, and significantly more slowly through single-stranded nucleic acid. Accordingly, this can serve as the basis of an assay. Thus, by adding ETMs (either covalently to one of the strands or non-covalently to the hybridization complex through the use of hybridization indicators, described below) to a nucleic acid that is attached to a detection electrode via a conductive oligomer, electron transfer between the ETM and the electrode, through the nucleic acid and conductive oligomer, may be detected. This general idea is depicted in Figure 27.

Alternatively, the ETM can be detected, not necessarily via electron transfer through nucleic acid, but rather can be directly detected using conductive oligomers; that is, the electrons from the ETMs need not travel through the stacked π orbitals in order to generate a signal. Instead, the presence of ETMs on the surface of a self-assembled monolayer (SAM), that comprises conductive oligomers, can be directly detected. This basic idea is termed "mechanism-2" herein. Thus, upon binding of a target sequence, a label probe comprising an ETM is brought to the surface, and detection of the ETM can proceed, putatively through the conductive oligomer to the electrode (alternatively, the target sequence comprises the ETMs). Essentially, the role of the SAM comprising the conductive oligomers is to "raise" the electronic surface of the electrode, while still providing the benefits of shielding the electrode from solution components and reducing the amount of non-specific binding to the electrodes. Viewed differently, the role of the target sequence is to provide specificity for a recruitment of ETMs to the surface, where they can be detected using conductive oligomers with electronically exposed termini. This general idea is shown in Figure 16.

Thus, in either embodiment, an assay complex is formed that contains an ETM, which is then detected using the detection electrode. The invention thus provides assay complexes that minimally comprise a target sequence. "Assay complex" herein is meant the collection of hybridization complexes comprising nucleic acids, including probes and targets, that contains at least one ETM and thus allows
5 detection. The composition of the assay complex depends on the use of the different probe components outlined herein. Thus, in Figures 16A and 16B, the assay complex comprises the capture probe and the target sequence. The assay complexes may also include label probes, capture extender probes, label extender probes, and amplifier probes, as outlined herein, depending on the configuration used.

10 The assay complexes comprise at least one ETM, which can either be covalently attached to a component of the assay complex as described herein or a "hybridization indicator", described below. The terms "electron donor moiety", "electron acceptor moiety", and "ETMs" (ETMs) or grammatical equivalents herein refers to molecules capable of electron transfer under certain conditions. It is to be understood that electron donor and acceptor capabilities are relative; that is, a molecule which can
15 lose an electron under certain experimental conditions will be able to accept an electron under different experimental conditions. It is to be understood that the number of possible electron donor moieties and electron acceptor moieties is very large, and that one skilled in the art of electron transfer compounds will be able to utilize a number of compounds in the present invention. Preferred ETMs include, but are not limited to, transition metal complexes, organic ETMs, and electrodes.

20 In a preferred embodiment, the ETMs are transition metal complexes. Transition metals are those whose atoms have a partial or complete d shell of electrons. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinum (Pt), scandium
25 (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinum, cobalt and iron.

30 The transition metals are complexed with a variety of ligands, to form suitable transition metal complexes, as is well known in the art. L are the co-ligands, that provide the coordination atoms for the binding of the metal ion. As will be appreciated by those in the art, the number and nature of the co-ligands will depend on the coordination number of the metal ion. Mono-, di- or polydentate co-ligands may be used at any position.

As will be appreciated in the art, the co-ligands can be the same or different. Suitable ligands fall into two categories: ligands which use nitrogen, oxygen, sulfur, carbon or phosphorus atoms (depending on the metal ion) as the coordination atoms (generally referred to in the literature as sigma (σ) donors) and organometallic ligands such as metallocene ligands (generally referred to in the literature as pi (π) donors, and depicted herein as L_m). Suitable nitrogen donating ligands are well known in the art and include, but are not limited to, NH_2 ; NHR ; NRR' ; pyridine; pyrazine; isonicotinamide; imidazole; bipyridine and substituted derivatives of bipyridine; terpyridine and substituted derivatives; phenanthrolines, particularly 1,10-phenanthroline (abbreviated phen) and substituted derivatives of phenanthrolines such as 4,7-dimethylphenanthroline and dipyrrol[3,2-a:2',3'-c]phenazine (abbreviated dppz); dipyrrophenazine; 1,4,5,8,9,12-hexaazatriphenylene (abbreviated hat); 9,10-phenanthrenequinone diimine (abbreviated phi); 1,4,5,8-tetraazaphenanthrene (abbreviated tap); 1,4,8,11-tetra-azacyclotetradecane (abbreviated cyclam), EDTA, EGTA and isocyanide. Substituted derivatives, including fused derivatives, may also be used. In some embodiments, porphyrins and substituted derivatives of the porphyrin family may be used. See for example, Comprehensive Coordination Chemistry, Ed. Wilkinson et al., Pergamon Press, 1987, Chapters 13.2 (pp73-98), 21.1 (pp. 813-898) and 21.3 (pp 915-957), all of which are hereby expressly incorporated by reference.

Suitable sigma donating ligands using carbon, oxygen, sulfur and phosphorus are known in the art. For example, suitable sigma carbon donors are found in Cotton and Wilkinson, Advanced Organic Chemistry, 5th Edition, John Wiley & Sons, 1988, hereby incorporated by reference; see page 38, for example. Similarly, suitable oxygen ligands include crown ethers, water and others known in the art. Phosphines and substituted phosphines are also suitable; see page 38 of Cotton and Wilkinson.

The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

In a preferred embodiment, organometallic ligands are used. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with δ -bonded organic ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π -bonded organic ligands (see Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich et al., 2nd Ed., 1992, VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel et al. Ed., Vol. 7, chapters 7, 8, 10 & 11, Pergamon Press, hereby expressly incorporated by reference). Such organometallic ligands include cyclic aromatic compounds such as the cyclopentadienide ion $[C_5H_5(-1)]$ and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadienyl) metal compounds, (i.e. the metallocenes); see for example Robins et al., J. Am. Chem. Soc.

104:1882-1893 (1982); and Gassman et al., J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by reference. Of these, ferrocene $[(C_5H_5)_2Fe]$ and its derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly et al., Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger et al., Advances in Organometallic Chemistry 23:1-93; and Geiger et al., Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row transition metals are potential candidates as redox moieties that are covalently attached to the nucleic acid. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example, Other acyclic π -bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic compounds, and all such ligands, in conjunction with other π -bonded and δ -bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties in nucleic acid analysis.

When one or more of the co-ligands is an organometallic ligand, the ligand is generally attached via one of the carbon atoms of the organometallic ligand, although attachment may be via other atoms for heterocyclic ligands. Preferred organometallic ligands include metallocene ligands, including substituted derivatives and the metalloceneophanes (see page 1174 of Cotton and Wilkinson, *supra*). For example, derivatives of metallocene ligands such as methylcyclopentadienyl, with multiple methyl groups being preferred, such as pentamethylcyclopentadienyl, can be used to increase the stability of the metallocene. In a preferred embodiment, only one of the two metallocene ligands of a metallocene are derivatized.

As described herein, any combination of ligands may be used. Preferred combinations include: a) all ligands are nitrogen donating ligands; b) all ligands are organometallic ligands; and c) one ligand is a metallocene ligand and another is a nitrogen donating ligand, with the other ligands, if needed, are either nitrogen donating ligands or metallocene ligands, or a mixture.

In addition to transition metal complexes, other organic electron donors and acceptors may be covalently attached to the nucleic acid for use in the invention. These organic molecules include, but are not limited to, riboflavin, xanthene dyes, azine dyes, acridine orange, *N,N*-dimethyl-2,7-diazapyrenium dichloride (DAP²⁺), methylviologen, ethidium bromide, quinones such as *N,N'*-dimethylantra(2,1,9-*def*:6,5,10-*d'e'f'*)diisoquinoline dichloride (ADIQ²⁺); porphyrins ([meso-tetrakis(*N*-methyl-*x*-pyridinium)porphyrin tetrachloride], varlamine blue B hydrochloride, Bindschedler's green;

2,6-dichloroindophenol, 2,6-dibromophenolindophenol; Brilliant crest blue (3-amino-9-dimethyl-amino-10-methylphenoxyazine chloride), methylene blue; Nile blue A (aminoaphthodiethylaminophenoxazine sulfate), indigo-5,5',7,7'-tetrasulfonic acid, indigo-5,5',7-trisulfonic acid; phenosafranine, indigo-5-monosulfonic acid; safranine T; bis(dimethylglyoximate)-iron(II) chloride; induline scarlet, neutral red, anthracene, coronene, pyrene, 9-phenylanthracene, rubrene, binaphthyl, DPA, phenothiazene, fluoranthene, phenanthrene, chrysene, 1,8-diphenyl-1,3,5,7-octatetracene, naphthalene, acenaphthalene, perylene, TMPD and analogs and substituted derivatives of these compounds.

In one embodiment, the electron donors and acceptors are redox proteins as are known in the art. However, redox proteins in many embodiments are not preferred.

10 The choice of the specific ETMs will be influenced by the type of electron transfer detection used, as is generally outlined below. Preferred ETMs are metallocenes, with ferrocene being particularly preferred.

In a preferred embodiment, a plurality of ETMs are used. The use of multiple ETMs provides signal amplification and thus allows more sensitive detection limits. While the use of multiple ETMs on nucleic acids that hybridize to complementary strands (i.e. mechanism-1 systems) can cause decreases in T_m s of the hybridization complexes depending on the number, site of attachment and spacing between the multiple ETMs, this is not a factor when the ETMs are on the recruitment linker, since this does not hybridize to a complementary sequence. Accordingly, pluralities of ETMs are preferred, with at least about 2 ETMs per assay complex being preferred, and at least about 10 being particularly preferred, and at least about 20 to 50 being especially preferred. In some instances, very large numbers of ETMs (100 to 10000 or greater) can be used.

Attachment of the ETM to the assay complex can be done in a wide variety of ways, and depends on the mechanism of detection and whether direct or indirect detection is done. In general, methods and compositions outlined in WO98/20162, expressly incorporated by reference in its entirety, can be used.

In a preferred embodiment, it is a label probe that comprises the ETMs. In a preferred embodiment, the label probe is used in a mechanism-2 detection system. Thus, as will be appreciated by those in the art, the portion of the label probe (or target, in some embodiments) that comprises the ETMs (termed herein a "recruitment linker" or "signal carrier") can be nucleic acid, or it can be a non-nucleic acid linker that links the first hybridizable portion of the label probe to the ETMs. That is, since this portion of the label probe is not required for hybridization if a mechanism-2 system is used, it need not

be nucleic acid, although this may be done for ease of synthesis. In some embodiments, as is more fully outlined below, the recruitment linker may comprise double-stranded portions.

Thus, as will be appreciated by those in the art, there are a variety of configurations that can be used. In a preferred embodiment, the recruitment linker is nucleic acid (including analogs), and attachment of the ETMs can be via (1) a base; (2) the backbone, including the ribose, the phosphate, or comparable structures in nucleic acid analogs; (3) nucleoside replacement, described below; or (4) metallocene polymers, as described below. In a preferred embodiment, the recruitment linker is non-nucleic acid, and can be either a metallocene polymer or an alkyl-type polymer (including heteroalkyl, as is more fully described below) containing ETM substitution groups. These options are generally depicted in the Figures.

When a mechanism-1 detection system is used to detect the label probes, the ETMs are generally attached via either (1) or (2) above, since hybridization of the label probe to a detection probe, as outlined herein, requires the formation of a hybridization complex.

In a preferred embodiment, the recruitment linker is a nucleic acid, and comprises covalently attached ETMs. The ETMs may be attached to nucleosides within the nucleic acid in a variety of positions. Preferred embodiments include, but are not limited to, (1) attachment to the base of the nucleoside, (2) attachment of the ETM as a base replacement, (3) attachment to the backbone of the nucleic acid, including either to a ribose of the ribose-phosphate backbone or to a phosphate moiety, or to analogous structures in nucleic acid analogs, and (4) attachment via metallocene polymers, with the latter being preferred.

In addition, as is described below, when the recruitment linker is nucleic acid, it may be desirable to use secondary label probes, that have a first portion that will hybridize to a portion of the primary label probes and a second portion comprising a recruitment linker as is defined herein. This is generally depicted in Figure 16H; this is similar to the use of an amplifier probe, except that both the primary and the secondary label probes comprise ETMs.

In a preferred embodiment, the ETM is attached to the base of a nucleoside as is generally outlined in WO 98/20162, incorporated by reference, for attachment of conductive oligomers. Attachment can be to an internal nucleoside or a terminal nucleoside.

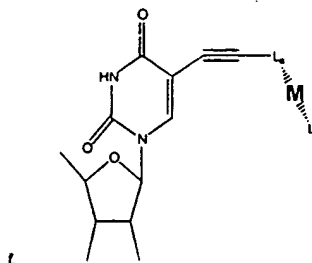
The covalent attachment to the base will depend in part on the ETM chosen, but in general is similar to the attachment of conductive oligomers to bases, as outlined in WO 98/20162. Attachment may generally be done to any position of the base. In a preferred embodiment, the ETM is a transition

metal complex, and thus attachment of a suitable metal ligand to the base leads to the covalent attachment of the ETM. Alternatively, similar types of linkages may be used for the attachment of organic ETMs, as will be appreciated by those in the art.

5 In one embodiment, the C4 attached amino group of cytosine, the C6 attached amino group of adenine, or the C2 attached amino group of guanine may be used as a transition metal ligand.

Ligands containing aromatic groups can be attached via acetylene linkages as is known in the art (see Comprehensive Organic Synthesis, Trost et al., Ed., Pergamon Press, Chapter 2.4: Coupling Reactions Between sp^2 and sp Carbon Centers, Sonogashira, pp521-549, and pp950-953, hereby incorporated by reference). Structure 1 depicts a representative structure in the presence of the metal ion and any other necessary ligands; Structure 1 depicts uridine, although as for all the structures herein, any other base may also be used.

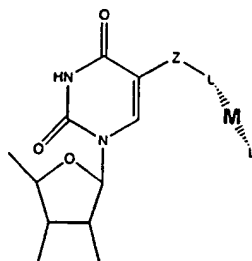
Structure 1



L_a is a ligand, which may include nitrogen, oxygen, sulfur or phosphorus donating ligands or organometallic ligands such as metallocene ligands. Suitable L_a ligands include, but not limited to, phenanthroline, imidazole, bpy and terpy. L_b and M are as defined above. Again, it will be appreciated by those in the art, a linker ("Z") may be included between the nucleoside and the ETM.

Similarly, as for the conductive oligomers, the linkage may be done using a linker, which may utilize an amide linkage (see generally Telser et al., J. Am. Chem. Soc. 111:7221-7226 (1989); Telser et al., J. Am. Chem. Soc. 111:7226-7232 (1989), both of which are expressly incorporated by reference). These structures are generally depicted below in Structure 2, which again uses uridine as the base, although as above, the other bases may also be used:

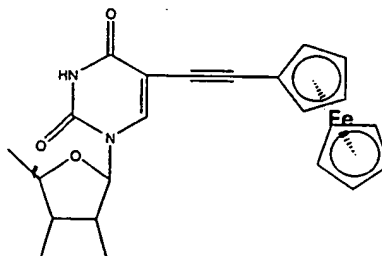
Structure 2



In this embodiment, L is a ligand as defined above, with L_r and M as defined above as well. Preferably, L is amino, phen, byp and terpy.

In a preferred embodiment, the ETM attached to a nucleoside is a metallocene; i.e. the L and L_r of Structure 2 are both metallocene ligands, L_m, as described above. Structure 3 depicts a preferred embodiment wherein the metallocene is ferrocene, and the base is uridine, although other bases may be used:

Structure 3



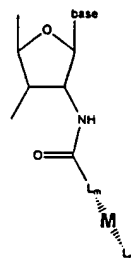
Preliminary data suggest that Structure 3 may cyclize, with the second acetylene carbon atom attacking the carbonyl oxygen, forming a furan-like structure. Preferred metallocenes include ferrocene, cobaltocene and osmiumocene.

In a preferred embodiment, the ETM is attached to a ribose at any position of the ribose-phosphate backbone of the nucleic acid, i.e. either the 5' or 3' terminus or any internal nucleoside. Ribose in this case can include ribose analogs. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose can be made, with nitrogen, oxygen, sulfur and phosphorus-containing modifications possible. Amino-modified and oxygen-modified ribose is preferred. See generally WO 95/15971 and WO 98/20162, incorporated herein by reference. These modification groups may be used as a transition metal ligand, or as a chemically functional moiety for attachment of other transition metal ligands and organometallic ligands, or organic electron donor moieties as will be appreciated by those in the art. In this embodiment, a linker such as depicted herein for "Z" may be used as well, or a conductive oligomer between the ribose and the ETM. Preferred embodiments utilize attachment at

the 2' or 3' position of the ribose, with the 2' position being preferred. Thus for example, the conductive oligomers depicted in Structure 13, 14 and 15 of WO98/20162 may be replaced by ETMs; alternatively, the ETMs may be added to the free terminus of the conductive oligomer.

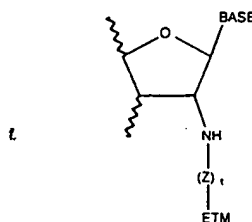
- 5 In a preferred embodiment, a metallocene serves as the ETM, and is attached via an amide bond as depicted below in Structure 4. The examples outline the synthesis of a preferred compound when the metallocene is ferrocene.

Structure 4



In a preferred embodiment, amine linkages are used, as is generally depicted in Structure 5.

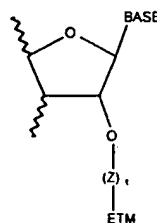
Structure 5



- 10 Z is a linker, as defined herein, with 1-16 atoms being preferred, and 2-4 atoms being particularly preferred, and t is either one or zero.

In a preferred embodiment, oxo linkages are used, as is generally depicted in Structure 6.

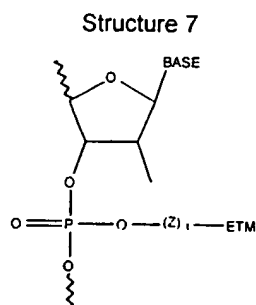
Structure 6



- 15 In Structure 6, Z is a linker, as defined herein, and t is either one or zero. Preferred Z linkers include alkyl groups including heteroalkyl groups such as $(CH_2)_n$ and $(CH_2CH_2O)_n$, with n from 1 to 10 being preferred, and n = 1 to 4 being especially preferred, and n=4 being particularly preferred.

Linkages utilizing other heteroatoms are also possible; a variety of linkages of ETMs to nucleosides are shown in Figure 1.

In a preferred embodiment, an ETM is attached to a phosphate at any position of the ribose-phosphate backbone of the nucleic acid. This may be done in a variety of ways. In one embodiment, phosphodiester bond analogs such as phosphoramidate or phosphoramidite linkages may be incorporated into a nucleic acid, where the heteroatom (i.e. nitrogen) serves as a transition metal ligand (see PCT publication WO 95/15971 and WO 98/20162, incorporated by reference). Alternatively, the conductive oligomers depicted in Structures 23 and 24 of WO98/20162 may be replaced by ETMs. In a preferred embodiment, the composition has the structure shown in Structure 7.



In Structure 7, the ETM is attached via a phosphate linkage, generally through the use of a linker, Z. Preferred Z linkers include alkyl groups, including heteroalkyl groups such as $(CH_2)_n$, $(CH_2CH_2O)_n$, with n from 1 to 10 being preferred, and n = 1 to 4 being especially preferred, and n=4 being particularly preferred.

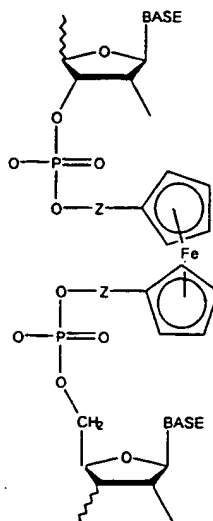
When the ETM is attached to the base or the backbone of the nucleoside, it is possible to attach the ETMs via "dendrimer" structures, as is more fully outlined below. As is generally depicted in the Figures, alkyl-based linkers can be used to create multiple branching structures comprising one or more ETMs at the terminus of each branch. Generally, this is done by creating branch points containing multiple hydroxy groups, which optionally can then be used to add additional branch points. The terminal hydroxy groups can then be used in phosphoramidite reactions to add ETMs, as is generally done below for the nucleoside replacement and metallocene polymer reactions.

In a preferred embodiment, an ETM such as a metallocene is used as a "nucleoside replacement", serving as an ETM. For example, the distance between the two cyclopentadiene rings of ferrocene is similar to the orthogonol distance between two bases in a double stranded nucleic acid. Other metallocenes in addition to ferrocene may be used, for example, air stable metallocenes such as those containing cobalt or ruthenium. Thus, metallocene moieties may be incorporated into the backbone of

a nucleic acid, as is generally depicted in Structure 8 (nucleic acid with a ribose-phosphate backbone) and Structure 9 (peptide nucleic acid backbone). Structures 8 and 9 depict ferrocene, although as will be appreciated by those in the art, other metallocenes may be used as well. In general, air stable metallocenes are preferred, including metallocenes utilizing ruthenium and cobalt as the metal.

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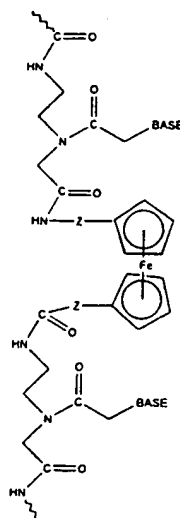
Structure 8



In Structure 8, Z is a linker as defined above, with generally short, alkyl groups, including heteroatoms such as oxygen being preferred. Generally, what is important is the length of the linker, such that minimal perturbations of a double stranded nucleic acid is effected, as is more fully described below. Thus, methylene, ethylene, ethylene glycols, propylene and butylene are all preferred, with ethylene and ethylene glycol being particularly preferred. In addition, each Z linker may be the same or different. Structure 8 depicts a ribose-phosphate backbone, although as will be appreciated by those in the art, nucleic acid analogs may also be used, including ribose analogs and phosphate bond analogs.

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Structure 9



In Structure 9, preferred Z groups are as listed above, and again, each Z linker can be the same or different. As above, other nucleic acid analogs may be used as well.

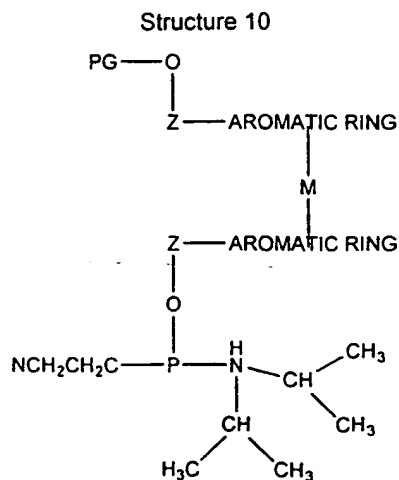
- In addition, although the structures and discussion above depicts metallocenes, and particularly ferrocene, this same general idea can be used to add ETMs in addition to metallocenes, as nucleoside replacements or in polymer embodiments, described below. Thus, for example, when the ETM is a transition metal complex other than a metallocene, comprising one, two or three (or more) ligands, the ligands can be functionalized as depicted for the ferrocene to allow the addition of phosphoramidite groups. Particularly preferred in this embodiment are complexes comprising at least two ring (for example, aryl and substituted aryl) ligands, where each of the ligands comprises functional groups for attachment via phosphoramidite chemistry. As will be appreciated by those in the art, this type of reaction, creating polymers of ETMs either as a portion of the backbone of the nucleic acid or as "side groups" of the nucleic acids, to allow amplification of the signals generated herein, can be done with virtually any ETM that can be functionalized to contain the correct chemical groups.
- Thus, by inserting a metallocene such as ferrocene (or other ETM) into the backbone of a nucleic acid, nucleic acid analogs are made; that is, the invention provides nucleic acids having a backbone comprising at least one metallocene. This is distinguished from nucleic acids having metallocenes attached to the backbone, i.e. via a ribose, a phosphate, etc. That is, two nucleic acids each made up of a traditional nucleic acid or analog (nucleic acids in this case including a single nucleoside), may be covalently attached to each other via a metallocene. Viewed differently, a metallocene derivative or substituted metallocene is provided, wherein each of the two aromatic rings of the metallocene has a nucleic acid substituent group.

In addition, as is more fully outlined below, it is possible to incorporate more than one metallocene into the backbone, either with nucleotides in between and/or with adjacent metallocenes. When adjacent metallocenes are added to the backbone, this is similar to the process described below as "metallocene polymers"; that is, there are areas of metallocene polymers within the backbone.

- 5 In addition to the nucleic acid substituent groups, it is also desirable in some instances to add additional substituent groups to one or both of the aromatic rings of the metallocene (or ETM). For example, as these nucleoside replacements are generally part of probe sequences to be hybridized with a substantially complementary nucleic acid, for example a target sequence or another probe sequence, it is possible to add substituent groups to the metallocene rings to facilitate hydrogen
- 10 bonding to the base or bases on the opposite strand. These may be added to any position on the metallocene rings. Suitable substituent groups include, but are not limited to, amide groups, amine groups, carboxylic acids, and alcohols, including substituted alcohols. In addition, these substituent groups can be attached via linkers as well, although in general this is not preferred.

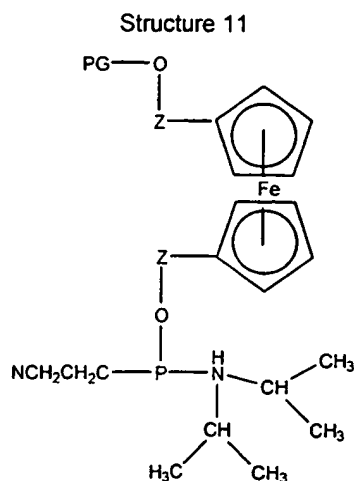
- 15 In addition, substituent groups on an ETM, particularly metallocenes such as ferrocene, may be added to alter the redox properties of the ETM. Thus, for example, in some embodiments, as is more fully described below, it may be desirable to have different ETMs attached in different ways (i.e. base or ribose attachment), on different probes, or for different purposes (for example, calibration or as an internal standard). Thus, the addition of substituent groups on the metallocene may allow two different ETMs to be distinguished.

- 20 In order to generate these metallocene-backbone nucleic acid analogs, the intermediate components are also provided. Thus, in a preferred embodiment, the invention provides phosphoramidite metallocenes, as generally depicted in Structure 10:



In Structure 10, PG is a protecting group, generally suitable for use in nucleic acid synthesis, with DMT, MMT and TMT all being preferred. The aromatic rings can either be the rings of the metallocene, or aromatic rings of ligands for transition metal complexes or other organic ETMs. The aromatic rings may be the same or different, and may be substituted as discussed herein.

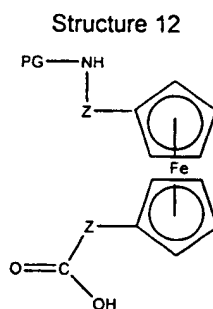
- 5 Structure 11 depicts the ferrocene derivative:



These phosphoramidite analogs can be added to standard oligonucleotide syntheses as is known in the art.

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- 10 Structure 12 depicts the ferrocene peptide nucleic acid (PNA) monomer, that can be added to PNA synthesis as is known in the art and depicted within the Figures and Examples:



In Structure 12, the PG protecting group is suitable for use in peptide nucleic acid synthesis, with MMT, boc and Fmoc being preferred.

- 15 These same intermediate compounds can be used to form ETM or metallocene polymers, which are added to the nucleic acids, rather than as backbone replacements, as is more fully described below.

In a preferred embodiment, the ETMs are attached as polymers, for example as metallocene polymers, in a "branched" configuration similar to the "branched DNA" embodiments herein and as outlined in U.S. Patent No. 5,124,246, using modified functionalized nucleotides. The general idea is as follows. A modified phosphoramidite nucleotide is generated that can ultimately contain a free hydroxy group that can be used in the attachment of phosphoramidite ETMs such as metallocenes. This free hydroxy group could be on the base or the backbone, such as the ribose or the phosphate (although as will be appreciated by those in the art, nucleic acid analogs containing other structures can also be used). The modified nucleotide is incorporated into a nucleic acid, and any hydroxy protecting groups are removed, thus leaving the free hydroxyl. Upon the addition of a phosphoramidite ETM such as a metallocene, as described above in structures 10 and 1, ETMs, such as metallocene ETMs, are added. Additional phosphoramidite ETMs such as metallocenes can be added, to form "ETM polymers", including "metallocene polymers" as depicted herein, particularly for ferrocene. In addition, in some embodiments, it is desirable to increase the solubility of the polymers by adding a "capping" group to the terminal ETM in the polymer, for example a final phosphate group to the metallocene as is generally depicted in Figure 12. Other suitable solubility enhancing "capping" groups will be appreciated by those in the art. It should be noted that these solubility enhancing groups can be added to the polymers in other places, including to the ligand rings, for example on the metallocenes as discussed herein

A preferred embodiment of this general idea is outlined in the Figures. In this embodiment, the 2' position of a ribose of a phosphoramidite nucleotide is first functionalized to contain a protected hydroxy group, in this case via an oxo-linkage, although any number of linkers can be used, as is generally described herein for Z linkers. The protected modified nucleotide is then incorporated via standard phosphoramidite chemistry into a growing nucleic acid. The protecting group is removed, and the free hydroxy group is used, again using standard phosphoramidite chemistry to add a phosphoramidite metallocene such as ferrocene. A similar reaction is possible for nucleic acid analogs. For example, using peptide nucleic acids and the metallocene monomer shown in Structure 12, peptide nucleic acid structures containing metallocene polymers could be generated.

Thus, the present invention provides recruitment linkers of nucleic acids comprising "branches" of metallocene polymers as is generally depicted in Figures 12 and 13. Preferred embodiments also utilize metallocene polymers from one to about 50 metallocenes in length, with from about 5 to about 20 being preferred and from about 5 to about 10 being especially preferred.

In addition, when the recruitment linker is nucleic acid, any combination of ETM attachments may be done.

In a preferred embodiment, the recruitment linker is not nucleic acid, and instead may be any sort of linker or polymer. As will be appreciated by those in the art, generally any linker or polymer that can be modified to contain ETMs can be used. In general, the polymers or linkers should be reasonably soluble and contain suitable functional groups for the addition of ETMs.

5 As used herein, a "recruitment polymer" comprises at least two or three subunits, which are covalently attached. At least some portion of the monomeric subunits contain functional groups for the covalent attachment of ETMs. In some embodiments coupling moieties are used to covalently link the subunits with the ETMs. Preferred functional groups for attachment are amino groups, carboxy groups, oxo groups and thiol groups, with amino groups being particularly preferred. As will be appreciated by those in the art, a wide variety of recruitment polymers are possible.

10 Suitable linkers include, but are not limited to, alkyl linkers (including heteroalkyl (including (poly)ethylene glycol-type structures), substituted alkyl, arylalkyl linkers, etc. As above for the polymers, the linkers will comprise one or more functional groups for the attachment of ETMs, which will be done as will be appreciated by those in the art, for example through the use homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference).

15 Suitable recruitment polymers include, but are not limited to, functionalized styrenes, such as amino styrene, functionalized dextrans, and polyamino acids. Preferred polymers are polyamino acids (both poly-D-amino acids and poly-L-amino acids), such as polylysine, and polymers containing lysine and other amino acids being particularly preferred. Other suitable polyamino acids are polyglutamic acid, polyaspartic acid, co-polymers of lysine and glutamic or aspartic acid, co-polymers of lysine with alanine, tyrosine, phenylalanine, serine, tryptophan, and/or proline.

In a preferred embodiment, the recruitment linker comprises a metallocene polymer, as is described above.

25 The attachment of the recruitment linkers to the first portion of the label probe will depend on the composition of the recruitment linker, as will be appreciated by those in the art. When the recruitment linker is nucleic acid, it is generally formed during the synthesis of the first portion of the label probe, with incorporation of nucleosides containing ETMs as required. Alternatively, the first portion of the label probe and the recruitment linker may be made separately, and then attached. For example, there may be an overlapping section of complementarity, forming a section of double stranded nucleic acid that can then be chemically crosslinked, for example by using psoralen as is known in the art.

When non-nucleic acid recruitment linkers are used, attachment of the linker/polymer of the recruitment linker will be done generally using standard chemical techniques, such as will be appreciated by those in the art. For example, when alkyl-based linkers are used, attachment can be similar to the attachment of insulators to nucleic acids.

5 In addition, it is possible to have recruitment linkers that are mixtures of nucleic acids and non-nucleic acids, either in a linear form (i.e. nucleic acid segments linked together with alkyl linkers) or in branched forms (nucleic acids with alkyl "branches" that may contain ETMs and may be additionally branched).

10 In a preferred embodiment, the ETM is attached to the target sequence, either by incorporation into a primer, or by incorporation into the newly synthesized portion of the target sequence. Target sequences comprising covalently attached ETMs can be directly detected using either a mechanism-1 or mechanism-2 system, as is shown in the figures.

15 In this embodiment, it is the target sequence itself that carries the ETMs, rather than the recruitment linker of a label probe. As discussed herein, this may be done using target sequences that have ETMs incorporated at any number of positions, including either within a primer or within the newly synthesized strand, and can be attached to the nucleic acid in a variety of positions, as outlined herein. In this embodiment, as for the others of the system, the 3'-5' orientation of the probes and targets is chosen to get the ETM-containing structures (i.e. label probes, recruitment linkers or target sequences) as close to the surface of the monolayer as possible, and in the correct orientation. This
20 may be done using attachment via insulators or conductive oligomers as is generally shown in the Figures, and may depend on which mechanism is used. In addition, as will be appreciated by those in the art, multiple capture probes can be utilized, either in a configuration wherein the 5'-3' orientation of the capture probes is different, or where "loops" of target form when multiples of capture probes are used.

25 Labelling of the target sequence with ETMs can also occur during synthesis of the new target strand. For example, as is described herein, it is possible to enzymatically add triphosphate nucleotides comprising the ETMs of the invention to a growing nucleic acid, for example during the previously described amplification techniques. As will be recognized by those in the art, while several enzymes have been shown to generally tolerate modified nucleotides, some of the modified nucleotides of the
30 invention, for example the "nucleoside replacement" embodiments and putatively some of the phosphate attachments, may or may not be recognized by the enzymes to allow incorporation into a growing nucleic acid. Therefore, preferred attachments in this embodiment are to the base or ribose of the nucleotide.

Alternatively, it is possible to enzymatically add nucleotides comprising ETMs to the terminus of a nucleic acid, for example a target nucleic acid, as is more fully outlined below. In this embodiment, an effective "recruitment linker" is added to the terminus of the target sequence, that can then be used for detection. Thus the invention provides compositions utilizing electrodes comprising monolayers of
5 conductive oligomers and capture probes, and target sequences that comprises a first portion that is capable of hybridizing to a component of an assay complex, and a second portion that does not hybridize to a component of an assay complex and comprises at least one covalently attached electron transfer moiety. Similarly, methods utilizing these compositions are also provided.

It is also possible to have ETMs connected to probe sequences, i.e. sequences designed to hybridize
10 to complementary sequences. Thus, ETMs may be added to non-recruitment linkers as well. For example, there may be ETMs added to sections of label probes that do hybridize to components of the assay complex, for example the first portion, or to the target sequence as outlined above. These ETMs may be used for electron transfer detection in some embodiments, or they may not, depending on the location and system. For example, in some embodiments, when for example the target sequence
15 containing randomly incorporated ETMs is hybridized directly to the capture probe, as is depicted in Figure 16A, there may be ETMs in the portion hybridizing to the capture probe. If the capture probe is attached to the electrode using a conductive oligomer, these ETMs can be used to detect electron transfer as has been previously described. Alternatively, these ETMs may not be specifically detected.

Similarly, in some embodiments, when the recruitment linker is nucleic acid, it may be desirable in
20 some instances to have some or all of the recruitment linker be double stranded. In one embodiment, there may be a second recruitment linker, substantially complementary to the first recruitment linker, that can hybridize to the first recruitment linker. In a preferred embodiment, the first recruitment linker comprises the covalently attached ETMs. In an alternative embodiment, the second recruitment linker
25 contains the ETMs, and the first recruitment linker does not, and the ETMs are recruited to the surface by hybridization of the second recruitment linker to the first. In yet another embodiment, both the first and second recruitment linkers comprise ETMs. It should be noted, as discussed above, that nucleic acids comprising a large number of ETMs may not hybridize as well, i.e. the T_m may be decreased, depending on the site of attachment and the characteristics of the ETM. Thus, in general, when
30 multiple ETMs are used on hybridizing strands, generally there are less than about 5, with less than about 3 being preferred, or alternatively the ETMs should be spaced sufficiently far apart that the intervening nucleotides can sufficiently hybridize to allow good kinetics.

In one embodiment, non-covalently attached ETMs may be used. In one embodiment, the ETM is a hybridization indicator. Hybridization indicators serve as an ETM that will preferentially associate with double stranded nucleic acid is added, usually reversibly, similar to the method of Millan et al., Anal. Chem. 65:2317-2323 (1993); Millan et al., Anal. Chem. 66:2943-2948 (1994), both of which are hereby
5 expressly incorporated by reference. In this embodiment, increases in the local concentration of ETMs, due to the association of the ETM hybridization indicator with double stranded nucleic acid at the surface, can be monitored using the monolayers comprising the conductive oligomers. Hybridization indicators include intercalators and minor and/or major groove binding moieties. In a preferred embodiment, intercalators may be used; since intercalation generally only occurs in the
10 presence of double stranded nucleic acid, only in the presence of double stranded nucleic acid will the ETMs concentrate. Intercalating transition metal complex ETMs are known in the art. Similarly, major or minor groove binding moieties, such as methylene blue, may also be used in this embodiment.

Similarly, the systems of the invention may utilize non-covalently attached ETMs, as is generally described in Napier et al., Bioconj. Chem. 8:906 (1997), hereby expressly incorporated by reference.
15 In this embodiment, changes in the redox state of certain molecules as a result of the presence of DNA (i.e. guanine oxidation by ruthenium complexes) can be detected using the SAMs comprising conductive oligomers as well.

Again, the configuration of the system will depend on the mechanism used for detection. A variety of mechanism-1 systems are depicted in Figure 27, which shows direct detection (i.e. target sequence
20 comprising the ETM), and indirect detection (using label probes).

A variety of mechanism-2 systems are depicted in Figure 16, again showing direct detection (i.e. target sequence comprising the ETM), and indirect detection (using label probes).

In a preferred embodiment, the label probes directly hybridize to the target sequences, as is generally depicted in the figures. In these embodiments, the target sequence is preferably, but not required to
25 be, immobilized on the surface using capture probes, including capture extender probes. Label probes are then used to bring the ETMs into proximity of the surface of the monolayer comprising conductive oligomers. In a preferred embodiment, multiple label probes are used; that is, label probes are designed such that the portion that hybridizes to the target sequence can be different for a number of different label probes, such that amplification of the signal occurs, since multiple label probes can
30 bind for every target sequence. Thus, as depicted in the figures, n is an integer of at least one. Depending on the sensitivity desired, the length of the target sequence, the number of ETMs per label probe, etc., preferred ranges of n are from 1 to 50, with from about 1 to about 20 being particularly preferred, and from about 2 to about 5 being especially preferred. In addition, if "generic" label probes

are desired, label extender probes can be used as generally described below for use with amplifier probes.

As above, generally in this embodiment the configuration of the system and the label probes are designed to recruit the ETMs as close as possible to the monolayer surface.

5 In a preferred embodiment, the label probes are hybridized to the target sequence indirectly. That is, the present invention finds use in novel combinations of signal amplification technologies and electron transfer detection on electrodes, which may be particularly useful in sandwich hybridization assays, as generally depicted in the Figures. In these embodiments, the amplifier probes of the invention are bound to the target sequence in a sample either directly or indirectly. Since the amplifier probes
10 preferably contain a relatively large number of amplification sequences that are available for binding of label probes, the detectable signal is significantly increased, and allows the detection limits of the target to be significantly improved. These label and amplifier probes, and the detection methods described herein, may be used in essentially any known nucleic acid hybridization formats, such as those in which the target is bound directly to a solid phase or in sandwich hybridization assays in
15 which the target is bound to one or more nucleic acids that are in turn bound to the solid phase.

The assay complexes of the invention are detected using electrodes.

By "electrode" herein is meant a composition, which, when connected to an electronic device, is able to sense a current or charge and convert it to a signal. Alternatively an electrode can be defined as a composition which can apply a potential to and/or pass electrons to or from species in the solution.
20 Thus, an electrode is an ETM as described herein. Preferred electrodes are known in the art and include, but are not limited to, certain metals and their oxides, including gold; platinum; palladium; silicon; aluminum; metal oxide electrodes including platinum oxide, titanium oxide, tin oxide, indium tin oxide, palladium oxide, silicon oxide, aluminum oxide, molybdenum oxide (Mo_2O_6), tungsten oxide (WO_3) and ruthenium oxides; and carbon (including glassy carbon electrodes, graphite and carbon
25 paste). Preferred electrodes include gold, silicon, platinum, carbon and metal oxide electrodes, with gold being particularly preferred.

The electrodes described herein are depicted as a flat surface, which is only one of the possible conformations of the electrode and is for schematic purposes only. The conformation of the electrode will vary with the detection method used. For example, flat planar electrodes may be preferred for
30 optical detection methods, or when arrays of nucleic acids are made, thus requiring addressable locations for both synthesis and detection. Alternatively, for single probe analysis, the electrode may be in the form of a tube, with the SAMs comprising conductive oligomers and nucleic acids bound to

the inner surface. Electrode coils may be preferred in some embodiments as well. This allows a maximum of surface area containing the nucleic acids to be exposed to a small volume of sample.

The detection electrode comprises a self-assembled monolayer (SAM) comprising conductive oligomers. By "monolayer" or "self-assembled monolayer" or "SAM" herein is meant a relatively ordered assembly of molecules spontaneously chemisorbed on a surface, in which the molecules are oriented approximately parallel to each other and roughly perpendicular to the surface. Each of the molecules includes a functional group that adheres to the surface, and a portion that interacts with neighboring molecules in the monolayer to form the relatively ordered array. A "mixed" monolayer comprises a heterogeneous monolayer, that is, where at least two different molecules make up the monolayer. The SAM may comprise conductive oligomers alone, or a mixture of conductive oligomers and insulators. As outlined herein, the efficiency of oligonucleotide hybridization may increase when the analyte is at a distance from the electrode. Similarly, non-specific binding of biomolecules, including the target analytes, to an electrode is generally reduced when a monolayer is present. Thus, a monolayer facilitates the maintenance of the nucleic acid away from the electrode surface. In addition, a monolayer serves to keep charged species away from the surface of the electrode. Thus, this layer helps to prevent electrical contact between the electrodes and the ETMs, or between the electrode and charged species within the solvent. Such contact can result in a direct "short circuit" or an indirect short circuit via charged species which may be present in the sample. Accordingly, the monolayer is preferably tightly packed in a uniform layer on the electrode surface, such that a minimum of "holes" exist. The monolayer thus serves as a physical barrier to block solvent accessibility to the electrode.

In a preferred embodiment, the monolayer comprises conductive oligomers. By "conductive oligomer" herein is meant a substantially conducting oligomer, preferably linear, some embodiments of which are referred to in the literature as "molecular wires". By "substantially conducting" herein is meant that the oligomer is capable of transferring electrons at 100 Hz. Generally, the conductive oligomer has substantially overlapping π -orbitals, i.e. conjugated π -orbitals, as between the monomeric units of the conductive oligomer, although the conductive oligomer may also contain one or more sigma (σ) bonds. Additionally, a conductive oligomer may be defined functionally by its ability to inject or receive electrons into or from an associated ETM. Furthermore, the conductive oligomer is more conductive than the insulators as defined herein. Additionally, the conductive oligomers of the invention are to be distinguished from electroactive polymers, that themselves may donate or accept electrons.

In a preferred embodiment, the conductive oligomers have a conductivity, S , of from between about 10^{-6} to about $10^4 \Omega^{-1}\text{cm}^{-1}$, with from about 10^{-5} to about $10^3 \Omega^{-1}\text{cm}^{-1}$ being preferred, with these S values being calculated for molecules ranging from about 20Å to about 200Å. As described below, insulators

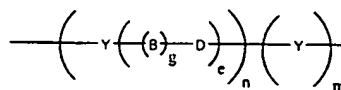
have a conductivity S of about $10^{-7} \Omega^{-1}\text{cm}^{-1}$ or lower, with less than about $10^{-8} \Omega^{-1}\text{cm}^{-1}$ being preferred. See generally Gardner et al., *Sensors and Actuators A* 51 (1995) 57-66, incorporated herein by reference.

Desired characteristics of a conductive oligomer include high conductivity, sufficient solubility in organic solvents and/or water for synthesis and use of the compositions of the invention, and preferably chemical resistance to reactions that occur i) during binding ligand synthesis (i.e. nucleic acid synthesis, such that nucleosides containing the conductive oligomers may be added to a nucleic acid synthesizer during the synthesis of the compositions of the invention, ii) during the attachment of the conductive oligomer to an electrode, or iii) during binding assays. In addition, conductive oligomers that will promote the formation of self-assembled monolayers are preferred.

The oligomers of the invention comprise at least two monomeric subunits, as described herein. As is described more fully below, oligomers include homo- and hetero-oligomers, and include polymers.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 13:

Structure 13



As will be understood by those in the art, all of the structures depicted herein may have additional atoms or structures; i.e. the conductive oligomer of Structure 1 may be attached to ETMs, such as electrodes, transition metal complexes, organic ETMs, and metallocenes, and to binding ligands such as nucleic acids, or to several of these. Unless otherwise noted, the conductive oligomers depicted herein will be attached at the left side to an electrode; that is, as depicted in Structure 1, the left "Y" is connected to the electrode as described herein. If the conductive oligomer is to be attached to a binding ligand, the right "Y", if present, is attached to the binding ligand such as a nucleic acid, either directly or through the use of a linker, as is described herein.

In this embodiment, Y is an aromatic group, n is an integer from 1 to 50, g is either 1 or zero, e is an integer from zero to 10, and m is zero or 1. When g is 1, B-D is a bond able to conjugate with neighboring bonds (herein referred to as a Aconjugated bond@), preferably selected from acetylene, alkene, substituted alkene, amide, azo, -C=N- (including -N=C-, -CR=N- and -N=CR-), -Si=Si-, and -Si=C- (including -C=Si-, -Si=CR- and -CR=Si-). When g is zero, e is preferably 1, D is preferably carbonyl, or a heteroatom moiety, wherein the heteroatom is selected from oxygen, sulfur, nitrogen, silicon or phosphorus. Thus, suitable heteroatom moieties include, but are not limited to, -NH and -NR, wherein R is as defined herein; substituted sulfur; sulfonyl (-SO₂-) sulfoxide (-SO-); phosphine

oxide (-PO- and -RPO-); and thiophosphine (-PS- and -RPS-). However, when the conductive oligomer is to be attached to a gold electrode, as outlined below, sulfur derivatives are not preferred.

By "aromatic group" or grammatical equivalents herein is meant an aromatic monocyclic or polycyclic hydrocarbon moiety generally containing 5 to 14 carbon atoms (although larger polycyclic rings structures may be made) and any carbocyclic ketone or thioketone derivative thereof, wherein the carbon atom with the free valence is a member of an aromatic ring. Aromatic groups include arylene groups and aromatic groups with more than two atoms removed. For the purposes of this application aromatic includes heterocycle. "Heterocycle" or "heteroaryl" means an aromatic group wherein 1 to 5 of the indicated carbon atoms are replaced by a heteroatom chosen from nitrogen, oxygen, sulfur, phosphorus, boron and silicon wherein the atom with the free valence is a member of an aromatic ring, and any heterocyclic ketone and thioketone derivative thereof. Thus, heterocycle includes thienyl, furyl, pyrrolyl, pyrimidinyl, oxalyl, indolyl, purinyl, quinolyl, isoquinolyl, thiazolyl, imidozyl, etc.

Importantly, the Y aromatic groups of the conductive oligomer may be different, i.e. the conductive oligomer may be a heterooligomer. That is, a conductive oligomer may comprise a oligomer of a single type of Y groups, or of multiple types of Y groups.

The aromatic group may be substituted with a substitution group, generally depicted herein as R. R groups may be added as necessary to affect the packing of the conductive oligomers, i.e. R groups may be used to alter the association of the oligomers in the monolayer. R groups may also be added to 1) alter the solubility of the oligomer or of compositions containing the oligomers; 2) alter the conjugation or electrochemical potential of the system; and 3) alter the charge or characteristics at the surface of the monolayer.

In a preferred embodiment, when the conductive oligomer is greater than three subunits, R groups are preferred to increase solubility when solution synthesis is done. However, the R groups, and their positions, are chosen to minimally effect the packing of the conductive oligomers on a surface, particularly within a monolayer, as described below. In general, only small R groups are used within the monolayer, with larger R groups generally above the surface of the monolayer. Thus for example the attachment of methyl groups to the portion of the conductive oligomer within the monolayer to increase solubility is preferred, with attachment of longer alkoxy groups, for example, C3 to C10, is preferably done above the monolayer surface. In general, for the systems described herein, this generally means that attachment of sterically significant R groups is not done on any of the first two or three oligomer subunits, depending on the average length of the molecules making up the monolayer.

Suitable R groups include, but are not limited to, hydrogen, alkyl, alcohol, aromatic, amino, amido, nitro, ethers, esters, aldehydes, sulfonyl, silicon moieties, halogens, sulfur containing moieties, phosphorus containing moieties, and ethylene glycols. In the structures depicted herein, R is hydrogen when the position is unsubstituted. It should be noted that some positions may allow two substitution groups, R and R', in which case the R and R' groups may be either the same or different.

By "alkyl group" or grammatical equivalents herein is meant a straight or branched chain alkyl group, with straight chain alkyl groups being preferred. If branched, it may be branched at one or more positions, and unless specified, at any position. The alkyl group may range from about 1 to about 30 carbon atoms (C1 -C30), with a preferred embodiment utilizing from about 1 to about 20 carbon atoms (C1 -C20), with about C1 through about C12 to about C15 being preferred, and C1 to C5 being particularly preferred, although in some embodiments the alkyl group may be much larger. Also included within the definition of an alkyl group are cycloalkyl groups such as C5 and C6 rings, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus. Alkyl also includes heteroalkyl, with heteroatoms of sulfur, oxygen, nitrogen, and silicone being preferred. Alkyl includes substituted alkyl groups. By "substituted alkyl group" herein is meant an alkyl group further comprising one or more substitution moieties "R", as defined above.

By "amino groups" or grammatical equivalents herein is meant -NH₂, -NHR and -NR₂ groups, with R being as defined herein.

By "nitro group" herein is meant an -NO₂ group.

By "sulfur containing moieties" herein is meant compounds containing sulfur atoms, including but not limited to, thia-, thio- and sulfo- compounds, thiols (-SH and -SR), and sulfides (-RSR-). By "phosphorus containing moieties" herein is meant compounds containing phosphorus, including, but not limited to, phosphines and phosphates. By "silicon containing moieties" herein is meant compounds containing silicon.

By "ether" herein is meant an -O-R group. Preferred ethers include alkoxy groups, with -O-(CH₂)₂CH₃ and -O-(CH₂)₄CH₃ being preferred.

By "ester" herein is meant a -COOR group.

By "halogen" herein is meant bromine, iodine, chlorine, or fluorine. Preferred substituted alkyls are partially or fully halogenated alkyls such as CF₃, etc.

By "aldehyde" herein is meant -RCHO groups.

By "alcohol" herein is meant -OH groups, and alkyl alcohols -ROH.

By "amido" herein is meant -RCONH- or RCONR- groups.

5 By "ethylene glycol" or "(poly)ethylene glycol" herein is meant a $-(O-CH_2-CH_2)_n-$ group, although each carbon atom of the ethylene group may also be singly or doubly substituted, i.e. $-(O-CR_2-CR_2)_n-$, with R as described above. Ethylene glycol derivatives with other heteroatoms in place of oxygen (i.e. $-(N-CH_2-CH_2)_n-$ or $-(S-CH_2-CH_2)_n-$, or with substitution groups) are also preferred.

Preferred substitution groups include, but are not limited to, methyl, ethyl, propyl, alkoxy groups such as $-O-(CH_2)_2CH_3$ and $-O-(CH_2)_4CH_3$ and ethylene glycol and derivatives thereof.

10 Preferred aromatic groups include, but are not limited to, phenyl, naphthyl, naphthalene, anthracene, phenanthroline, pyrrole, pyridine, thiophene, porphyrins, and substituted derivatives of each of these, included fused ring derivatives.

15 In the conductive oligomers depicted herein, when g is 1, B-D is a bond linking two atoms or chemical moieties. In a preferred embodiment, B-D is a conjugated bond, containing overlapping or conjugated π -orbitals.

Preferred B-D bonds are selected from acetylene ($-C\equiv C-$, also called alkyne or ethyne), alkene ($-CH=CH-$, also called ethylene), substituted alkene ($-CR=CR-$, $-CH=CR-$ and $-CR=CH-$), amide ($-NH-CO-$ and $-NR-CO-$ or $-CO-NH-$ and $-CO-NR-$), azo ($-N=N-$), esters and thioesters ($-CO-O-$, $-O-CO-$, $-CS-O-$ and $-O-CS-$) and other conjugated bonds such as ($-CH=N-$, $-CR=N-$, $-N=CH-$ and $-N=CR-$), ($-SiH=SiH-$, $-SiR=SiH-$, $-SiR=SiH-$, and $-SiR=SiR-$), ($-SiH=CH-$, $-SiR=CH-$, $-SiH=CR-$, $-SiR=CR-$, $-CH=SiH-$, $-CR=SiH-$, $-CH=SiR-$, and $-CR=SiR-$). Particularly preferred B-D bonds are acetylene, alkene, amide, and substituted derivatives of these three, and azo. Especially preferred B-D bonds are acetylene, alkene and amide. The oligomer components attached to double bonds may be in the trans or cis conformation, or mixtures. Thus, either B or D may include carbon, nitrogen or silicon.

25 The substitution groups are as defined as above for R.

When $g=0$ in the Structure 1 conductive oligomer, e is preferably 1 and the D moiety may be carbonyl or a heteroatom moiety as defined above.

As above for the Y rings, within any single conductive oligomer, the B-D bonds (or D moieties, when $g=0$) may be all the same, or at least one may be different. For example, when m is zero, the terminal B-D bond may be an amide bond, and the rest of the B-D bonds may be acetylene bonds. Generally, when amide bonds are present, as few amide bonds as possible are preferable, but in some
5 embodiments all the B-D bonds are amide bonds. Thus, as outlined above for the Y rings, one type of B-D bond may be present in the conductive oligomer within a monolayer as described below, and another type above the monolayer level, for example to give greater flexibility for nucleic acid hybridization when the nucleic acid is attached via a conductive oligomer.

10 In the structures depicted herein, n is an integer from 1 to 50, although longer oligomers may also be used (see for example Schumm et al., *Angew. Chem. Int. Ed. Engl.* 1994 33(13):1360). Without being bound by theory, it appears that for efficient hybridization of nucleic acids on a surface, the hybridization should occur at a distance from the surface, i.e. the kinetics of hybridization increase as a function of the distance from the surface, particularly for long oligonucleotides of 200 to 300
15 basepairs. Accordingly, when a nucleic acid is attached via a conductive oligomer, as is more fully described below, the length of the conductive oligomer is such that the closest nucleotide of the nucleic acid is positioned from about 6Å to about 100Å (although distances of up to 500Å may be used) from the electrode surface, with from about 15Å to about 60Å being preferred and from about 25Å to about 60Å also being preferred. Accordingly, n will depend on the size of the aromatic group, but generally will be from about 1 to about 20, with from about 2 to about 15 being preferred and from
20 about 3 to about 10 being especially preferred.

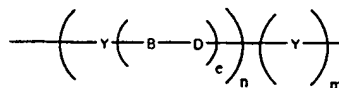
In the structures depicted herein, m is either 0 or 1. That is, when m is 0, the conductive oligomer may terminate in the B-D bond or D moiety, i.e. the D atom is attached to the nucleic acid either directly or via a linker. In some embodiments, for example when the conductive oligomer is attached to a
25 phosphate of the ribose-phosphate backbone of a nucleic acid, there may be additional atoms, such as a linker, attached between the conductive oligomer and the nucleic acid. Additionally, as outlined below, the D atom may be the nitrogen atom of the amino-modified ribose. Alternatively, when m is 1, the conductive oligomer may terminate in Y, an aromatic group, i.e. the aromatic group is attached to the nucleic acid or linker.

As will be appreciated by those in the art, a large number of possible conductive oligomers may be
30 utilized. These include conductive oligomers falling within the Structure 1 and Structure 8 formulas, as well as other conductive oligomers, as are generally known in the art, including for example, compounds comprising fused aromatic rings or Teflon®-like oligomers, such as $-(CF_2)_n-$, $-(CHF)_n-$ and $-(CFR)_n-$. See for example, Schumm et al., *Angew. Chem. Intl. Ed. Engl.* 33:1361 (1994); Grosshenny et al., *Platinum Metals Rev.* 40(1):26-35 (1996); Tour, *Chem. Rev.* 96:537-553 (1996); Hsung et al.,

Organometallics 14:4808-4815 (1995; and references cited therein, all of which are expressly incorporated by reference.

Particularly preferred conductive oligomers of this embodiment are depicted below:

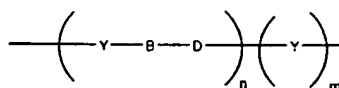
Structure 14



- 5 Structure 14 is Structure 13 when g is 1. Preferred embodiments of Structure 14 include: e is zero, Y is pyrrole or substituted pyrrole; e is zero, Y is thiophene or substituted thiophene; e is zero, Y is furan or substituted furan; e is zero, Y is phenyl or substituted phenyl; e is zero, Y is pyridine or substituted pyridine; e is 1, B-D is acetylene and Y is phenyl or substituted phenyl (see Structure 16 below). A preferred embodiment of Structure 14 is also when e is one, depicted as Structure 15 below:

10

Structure 15

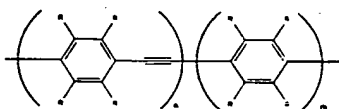


- Preferred embodiments of Structure 15 are: Y is phenyl or substituted phenyl and B-D is azo; Y is phenyl or substituted phenyl and B-D is acetylene; Y is phenyl or substituted phenyl and B-D is alkene; Y is pyridine or substituted pyridine and B-D is acetylene; Y is thiophene or substituted thiophene and B-D is acetylene; Y is furan or substituted furan and B-D is acetylene; Y is thiophene or furan (or substituted thiophene or furan) and B-D are alternating alkene and acetylene bonds.
- 15

Most of the structures depicted herein utilize a Structure 15 conductive oligomer. However, any Structure 15 oligomers may be substituted with any of the other structures depicted herein, i.e. Structure 13 or 20 oligomer, or other conducting oligomer, and the use of such Structure 15 depiction is not meant to limit the scope of the invention.

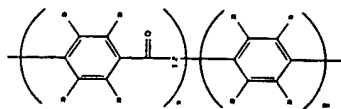
- 20 Particularly preferred embodiments of Structure 15 include Structures 16, 17, 18 and 19, depicted below:

Structure 16



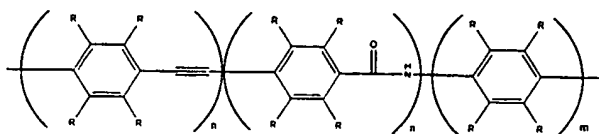
Particularly preferred embodiments of Structure 16 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen; and the use of R groups to increase solubility.

Structure 17



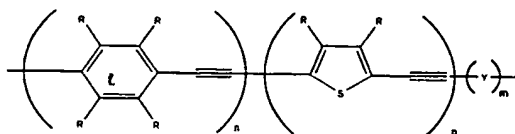
When the B-D bond is an amide bond, as in Structure 17, the conductive oligomers are pseudopeptide oligomers. Although the amide bond in Structure 17 is depicted with the carbonyl to the left, i.e. -CONH-, the reverse may also be used, i.e. -NHCO-. Particularly preferred embodiments of Structure 17 include: n is two, m is one, and R is hydrogen; n is three, m is zero, and R is hydrogen (in this embodiment, the terminal nitrogen (the D atom) may be the nitrogen of the amino-modified ribose); and the use of R groups to increase solubility.

Structure 18



Preferred embodiments of Structure 18 include the first n is two, second n is one, m is zero, and all R groups are hydrogen, or the use of R groups to increase solubility.

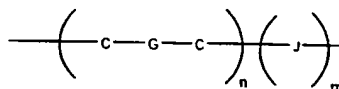
Structure 19



Preferred embodiments of Structure 19 include: the first n is three, the second n is from 1-3, with m being either 0 or 1, and the use of R groups to increase solubility.

In a preferred embodiment, the conductive oligomer has the structure depicted in Structure 20:

Structure 20



In this embodiment, C are carbon atoms, n is an integer from 1 to 50, m is 0 or 1, J is a heteroatom selected from the group consisting of oxygen, nitrogen, silicon, phosphorus, sulfur, carbonyl or sulfoxide, and G is a bond selected from alkane, alkene or acetylene, such that together with the two carbon atoms the C-G-C group is an alkene (-CH=CH-), substituted alkene (-CR=CR-) or mixtures thereof (-CH=CR- or -CR=CH-), acetylene (-C≡C-), or alkane (-CR₂-CR₂-, with R being either hydrogen or a substitution group as described herein). The G bond of each subunit may be the same or different than the G bonds of other subunits; that is, alternating oligomers of alkene and acetylene

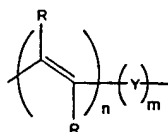
bonds could be used, etc. However, when G is an alkane bond, the number of alkane bonds in the oligomer should be kept to a minimum, with about six or less sigma bonds per conductive oligomer being preferred. Alkene bonds are preferred, and are generally depicted herein, although alkane and acetylene bonds may be substituted in any structure or embodiment described herein as will be appreciated by those in the art.

In some embodiments, for example when ETMs are not present, if $m=0$ then at least one of the G bonds is not an alkane bond.

In a preferred embodiment, the m of Structure 20 is zero. In a particularly preferred embodiment, m is zero and G is an alkene bond, as is depicted in Structure 21:

10

Structure 21



The alkene oligomer of structure 21, and others depicted herein, are generally depicted in the preferred trans configuration, although oligomers of cis or mixtures of trans and cis may also be used. As above, R groups may be added to alter the packing of the compositions on an electrode, the hydrophilicity or hydrophobicity of the oligomer, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the oligomer. n is as defined above.

In a preferred embodiment, R is hydrogen, although R may be also alkyl groups and polyethylene glycols or derivatives.

In an alternative embodiment, the conductive oligomer may be a mixture of different types of oligomers, for example of structures 13 and 20.

In addition, particularly for use with mechanism-2 systems, the monolayer comprises conductive oligomers, and the terminus of at least some of the conductive oligomers in the monolayer are electronically exposed. By "electronically exposed" herein is meant that upon the placement of an ETM in close proximity to the terminus, and after initiation with the appropriate signal, a signal dependent on the presence of the ETM may be detected. The conductive oligomers may or may not have terminal groups. Thus, in a preferred embodiment, there is no additional terminal group, and the conductive oligomer terminates with one of the groups depicted in the structures; for example, a B-D bond such as an acetylene bond. Alternatively, in a preferred embodiment, a terminal group is added, sometimes depicted herein as "Q". A terminal group may be used for several reasons; for example, to

contribute to the electronic availability of the conductive oligomer for detection of ETMs, or to alter the surface of the SAM for other reasons, for example to prevent non-specific binding. For example, when the target analyte is a nucleic acid, there may be negatively charged groups on the terminus to form a negatively charged surface such that when the nucleic acid is DNA or RNA the nucleic acid is repelled or prevented from lying down on the surface, to facilitate hybridization. Preferred terminal groups include -NH₂, -OH, -COOH, and alkyl groups such as -CH₃, and (poly)alkyloxides such as (poly)ethylene glycol, with -OCH₂CH₂OH, -(OCH₂CH₂O)₂H, -(OCH₂CH₂O)₃H, and -(OCH₂CH₂O)₄H being preferred.

In one embodiment, it is possible to use mixtures of conductive oligomers with different types of terminal groups. Thus, for example, some of the terminal groups may facilitate detection, and some may prevent non-specific binding.

It will be appreciated that the monolayer may comprise different conductive oligomer species, although preferably the different species are chosen such that a reasonably uniform SAM can be formed. Thus, for example, when capture binding ligands such as nucleic acids are covalently attached to the electrode using conductive oligomers, it is possible to have one type of conductive oligomer used to attach the nucleic acid, and another type functioning to detect the ETM. Similarly, it may be desirable to have mixtures of different lengths of conductive oligomers in the monolayer, to help reduce non-specific signals. Thus, for example, preferred embodiments utilize conductive oligomers that terminate below the surface of the rest of the monolayer, i.e. below the insulator layer, if used, or below some fraction of the other conductive oligomers. Similarly, the use of different conductive oligomers may be done to facilitate monolayer formation, or to make monolayers with altered properties.

In a preferred embodiment, the monolayer may further comprise insulator moieties. By "insulator" herein is meant a substantially nonconducting oligomer, preferably linear. By "substantially nonconducting" herein is meant that the insulator will not transfer electrons at 100 Hz. The rate of electron transfer through the insulator is preferably slower than the rate through the conductive oligomers described herein.

In a preferred embodiment, the insulators have a conductivity, S , of about $10^{-7} \Omega^{-1}\text{cm}^{-1}$ or lower, with less than about $10^{-8} \Omega^{-1}\text{cm}^{-1}$ being preferred. See generally Gardner et al., *supra*.

Generally, insulators are alkyl or heteroalkyl oligomers or moieties with sigma bonds, although any particular insulator molecule may contain aromatic groups or one or more conjugated bonds. By "heteroalkyl" herein is meant an alkyl group that has at least one heteroatom, i.e. nitrogen, oxygen, sulfur, phosphorus, silicon or boron included in the chain. Alternatively, the insulator may be quite

similar to a conductive oligomer with the addition of one or more heteroatoms or bonds that serve to inhibit or slow, preferably substantially, electron transfer.

Suitable insulators are known in the art, and include, but are not limited to, $-(CH_2)_n-$, $-(CRH)_n-$, and $-(CR_2)_n-$, ethylene glycol or derivatives using other heteroatoms in place of oxygen, i.e. nitrogen or sulfur (sulfur derivatives are not preferred when the electrode is gold).

As for the conductive oligomers, the insulators may be substituted with R groups as defined herein to alter the packing of the moieties or conductive oligomers on an electrode, the hydrophilicity or hydrophobicity of the insulator, and the flexibility, i.e. the rotational, torsional or longitudinal flexibility of the insulator. For example, branched alkyl groups may be used. Similarly, the insulators may contain terminal groups, as outlined above, particularly to influence the surface of the monolayer.

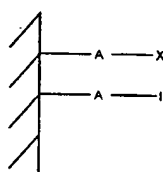
The length of the species making up the monolayer will vary as needed. As outlined above, it appears that binding of target analytes (for example, hybridization of nucleic acids) is more efficient at a distance from the surface. The species to which capture binding ligands are attached (as outlined below, these can be either insulators or conductive oligomers) may be basically the same length as the monolayer forming species or longer than them, resulting in the capture binding ligands being more accessible to the solvent for hybridization. In some embodiments, the conductive oligomers to which the capture binding ligands are attached may be shorter than the monolayer.

As will be appreciated by those in the art, the actual combinations and ratios of the different species making up the monolayer can vary widely, and will depend on whether mechanism-1 or -2 is used. Generally, three component systems are preferred for mechanism-2 systems, with the first species comprising a capture binding ligand containing species (termed a capture probe when the target analyte is a nucleic acid), attached to the electrode via either an insulator or a conductive oligomer. The second species are the conductive oligomers, and the third species are insulators. In this embodiment, the first species can comprise from about 90% to about 1%, with from about 20% to about 40% being preferred. When the target analytes are nucleic acids, from about 30% to about 40% is especially preferred for short oligonucleotide targets and from about 10% to about 20% is preferred for longer targets. The second species can comprise from about 1% to about 90%, with from about 20% to about 90% being preferred, and from about 40% to about 60% being especially preferred. The third species can comprise from about 1% to about 90%, with from about 20% to about 40% being preferred, and from about 15% to about 30% being especially preferred. Preferred ratios of first:second:third species are 2:2:1 for short targets, 1:3:1 for longer targets, with total thiol concentration (when used to attach these species, as is more fully outlined below) in the 500 μ M to 1 mM range, and 833 μ M being preferred.

Alternatively, two component systems can be used. In one embodiment, for use in either mechanism-1 or mechanism-2 systems, the two components are the first and second species. In this embodiment, the first species can comprise from about 1% to about 90%, with from about 1% to about 40% being preferred, and from about 10% to about 40% being especially preferred. The second species can comprise from about 1% to about 90%, with from about 10% to about 60% being preferred, and from about 20% to about 40% being especially preferred. Alternatively, for mechanism-1 systems, the two components are the first and the third species. In this embodiment, the first species can comprise from about 1% to about 90%, with from about 1% to about 40% being preferred, and from about 10% to about 40% being especially preferred. The second species can comprise from about 1% to about 90%, with from about 10% to about 60% being preferred, and from about 20% to about 40% being especially preferred.

The covalent attachment of the conductive oligomers and insulators to the electrode may be accomplished in a variety of ways, depending on the electrode and the composition of the insulators and conductive oligomers used. The attachment linkers (which can comprise insulators and conductive oligomers), which are used to covalently attach nucleic acid species (capture and detection probes) to the electrode, are attached in a similar manner. Thus, one end or terminus of the attachment linker is attached to the nucleoside or nucleic acid, and the other is attached to an electrode. In some embodiments it may be desirable to have the attachment linker attached at a position other than a terminus, or even to have a branched attachment linker that is attached to an electrode at one terminus and to two or more nucleosides at other termini, although this is not preferred. Similarly, the attachment linker may be attached at two sites to the electrode, as is generally depicted in Structures 23-25. Generally, some type of linker is used, as depicted below as "A" in Structure 22, where "X" is the conductive oligomer, "I" is an insulator and the hatched surface is the electrode:

Structure 22

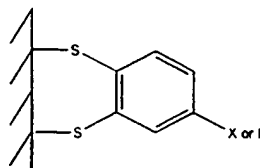


In this embodiment, A is a linker or atom. The choice of "A" will depend in part on the characteristics of the electrode. Thus, for example, A may be a sulfur moiety when a gold electrode is used. Alternatively, when metal oxide electrodes are used, A may be a silicon (silane) moiety attached to the oxygen of the oxide (see for example Chen et al., *Langmuir* 10:3332-3337 (1994); Lenhard et al., *J. Electroanal. Chem.* 78:195-201 (1977), both of which are expressly incorporated by reference). When carbon based electrodes are used, A may be an amino moiety (preferably a primary amine; see for

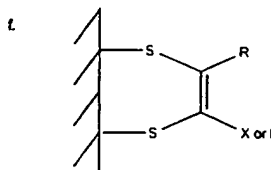
example Deinhammer et al., Langmuir 10:1306-1313 (1994)). Thus, preferred A moieties include, but are not limited to, silane moieties, sulfur moieties (including alkyl sulfur moieties), and amino moieties. In a preferred embodiment, epoxide type linkages with redox polymers such as are known in the art are not used.

- 5 Although depicted herein as a single moiety, the insulators and conductive oligomers may be attached to the electrode with more than one "A" moiety; the "A" moieties may be the same or different. Thus, for example, when the electrode is a gold electrode, and "A" is a sulfur atom or moiety, multiple sulfur atoms may be used to attach the conductive oligomer to the electrode, such as is generally depicted below in Structures 23, 24 and 25. As will be appreciated by those in the art, other such structures
- 10 can be made. In Structures 23, 24 and 25, the A moiety is just a sulfur atom, but substituted sulfur moieties may also be used.

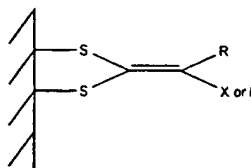
Structure 23



Structure 24



Structure 25

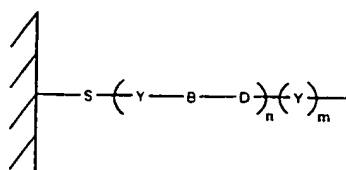


- 15 It should also be noted that similar to Structure 25, it may be possible to have a conductive oligomer terminating in a single carbon atom with three sulfur moieties attached to the electrode. Additionally, although not always depicted herein, the conductive oligomers and insulators may also comprise a "Q" terminal group.

- 20 In a preferred embodiment, the electrode is a gold electrode, and attachment is via a sulfur linkage as is well known in the art, i.e. the A moiety is a sulfur atom or moiety. Although the exact characteristics

of the gold-sulfur attachment are not known, this linkage is considered covalent for the purposes of this invention. A representative structure is depicted in Structure 26, using the Structure 15 conductive oligomer, although as for all the structures depicted herein, any of the conductive oligomers, or combinations of conductive oligomers, may be used. Similarly, any of the conductive oligomers or insulators may also comprise terminal groups as described herein. Structure 26 depicts the "A" linker as comprising just a sulfur atom, although additional atoms may be present (i.e. linkers from the sulfur to the conductive oligomer or substitution groups). In addition, Structure 26 shows the sulfur atom attached to the Y aromatic group, but as will be appreciated by those in the art, it may be attached to the B-D group (i.e. an acetylene) as well.

Structure 26

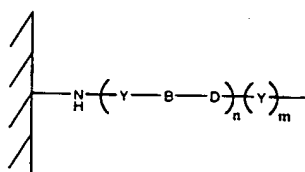


In general, thiol linkages are preferred when either two sets of electrodes are used (i.e. the detection electrodes comprising the SAMs are not used at high electrophoretic voltages (i.e. greater than 800 or 900 mV), that can cause oxidation of the thiol linkage and thus loss of the SAM), or, if one set of electrodes is used, lower electrophoretic voltages are used as is generally described below.

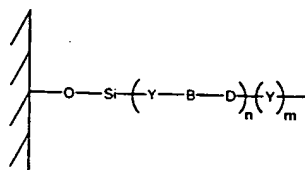
t

In a preferred embodiment, the electrode is a carbon electrode, i.e. a glassy carbon electrode, and attachment is via a nitrogen of an amine group. A representative structure is depicted in Structure 27. Again, additional atoms may be present, i.e. Z type linkers and/or terminal groups.

Structure 27



Structure 28



In Structure 28, the oxygen atom is from the oxide of the metal oxide electrode. The Si atom may also contain other atoms, i.e. be a silicon moiety containing substitution groups. Other attachments for

SAMs to other electrodes are known in the art; see for example Napier et al., Langmuir, 1997, for attachment to indium tin oxide electrodes, and also the chemisorption of phosphates to an indium tin oxide electrode (talk by H. Holden Thorpe, CHI conference, May 4-5, 1998).

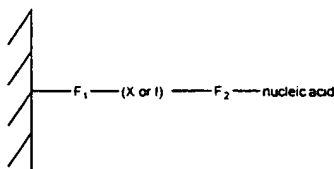
5 In a preferred embodiment, the electrode comprises either capture probes to anchor the assay complexes to the electrode (used in either mechanism-1 or mechanism-2 systems), or detection probes (for mechanism-1 systems). Since the capture probes are not used for detection, the capture probes may be attached using attachment linkers, which may include either conductive oligomers or insulators, as described below. Detection probes used in mechanism-1 systems are attached using conductive oligomers.

10 The capture probe nucleic acid is covalently attached to the electrode, via an "attachment linker", that can be either a conductive oligomer (required for mechanism-1 systems) or an insulator. By "covalently attached" herein is meant that two moieties are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds.

15 Thus, one end of the attachment linker is attached to a nucleic acid (or other binding ligand), and the other end (although as will be appreciated by those in the art, it need not be the exact terminus for either) is attached to the electrode. Thus, any of structures depicted herein may further comprise a nucleic acid effectively as a terminal group. Thus, the present invention provides compositions comprising nucleic acids covalently attached to electrodes as is generally depicted below in Structure 29:

20

Structure 29



25

In Structure 29, the hatched marks on the left represent an electrode. X is a conductive oligomer and I is an insulator as defined herein. F₁ is a linkage that allows the covalent attachment of the electrode and the conductive oligomer or insulator, including bonds, atoms or linkers such as is described herein, for example as "A", defined below. F₂ is a linkage that allows the covalent attachment of the conductive oligomer or insulator to the nucleic acid, and may be a bond, an atom or a linkage as is herein described. F₂ may be part of the conductive oligomer, part of the insulator, part of the nucleic acid, or exogenous to both, for example, as defined herein for "Z".

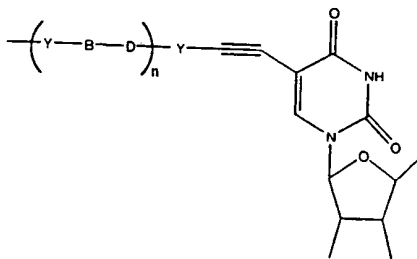
In a preferred embodiment, the capture probe nucleic acid is covalently attached to the electrode via a conductive oligomer. The covalent attachment of the nucleic acid and the conductive oligomer may be accomplished in several ways. In a preferred embodiment, the attachment is via attachment to the base of the nucleoside, via attachment to the backbone of the nucleic acid (either the ribose, the phosphate, or to an analogous group of a nucleic acid analog backbone), or via a transition metal ligand, as described below. The techniques outlined below are generally described for naturally occurring nucleic acids, although as will be appreciated by those in the art, similar techniques may be used with nucleic acid analogs, and in some cases with other binding ligands.

In a preferred embodiment, the conductive oligomer is attached to the base of a nucleoside of the nucleic acid. This may be done in several ways, depending on the oligomer, as is described below. In one embodiment, the oligomer is attached to a terminal nucleoside, i.e. either the 3' or 5' nucleoside of the nucleic acid. Alternatively, the conductive oligomer is attached to an internal nucleoside.

The point of attachment to the base will vary with the base. Generally, attachment at any position is possible. In some embodiments, for example when the probe containing the ETMs may be used for hybridization (i.e. mechanism-1 systems), it is preferred to attach at positions not involved in hydrogen bonding to the complementary base. Thus, for example, generally attachment is to the 5 or 6 position of pyrimidines such as uridine, cytosine and thymine. For purines such as adenine and guanine, the linkage is preferably via the 8 position. Attachment to non-standard bases is preferably done at the comparable positions.

In one embodiment, the attachment is direct; that is, there are no intervening atoms between the conductive oligomer and the base. In this embodiment, for example, conductive oligomers with terminal acetylene bonds are attached directly to the base. Structure 30 is an example of this linkage, using a Structure 15 conductive oligomer and uridine as the base, although other bases and conductive oligomers can be used as will be appreciated by those in the art:

Structure 30



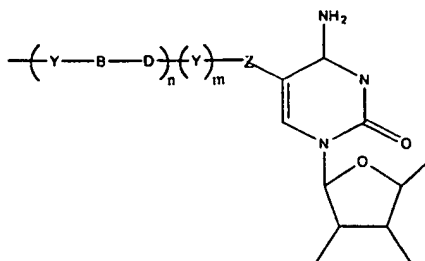
It should be noted that the pentose structures depicted herein may have hydrogen, hydroxy, phosphates or other groups such as amino groups attached. In addition, the pentose and nucleoside

structures depicted herein are depicted non-conventionally, as mirror images of the normal rendering. In addition, the pentose and nucleoside structures may also contain additional groups, such as protecting groups, at any position, for example as needed during synthesis.

In addition, the base may contain additional modifications as needed, i.e. the carbonyl or amine groups may be altered or protected.

In an alternative embodiment, the attachment is any number of different Z linkers, including amide and amine linkages, as is generally depicted in Structure 31 using uridine as the base and a Structure 15 oligomer:

Structure 31:



In this embodiment, Z is a linker. Preferably, Z is a short linker of about 1 to about 10 atoms, with from 1 to 5 atoms being preferred, that may or may not contain alkene, alkynyl, amine, amide, azo, imine, etc., bonds. Linkers are known in the art; for example, homo- or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Preferred Z linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups, esters, amide, amine, epoxy groups and ethylene glycol and derivatives being preferred, with propyl, acetylene, and C₂ alkene being especially preferred. Z may also be a sulfone group, forming sulfonamide linkages as discussed below.

In a preferred embodiment, the attachment of the nucleic acid and the conductive oligomer is done via attachment to the backbone of the nucleic acid. This may be done in a number of ways, including attachment to a ribose of the ribose-phosphate backbone, or to the phosphate of the backbone, or other groups of analogous backbones.

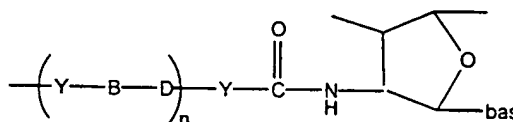
As a preliminary matter, it should be understood that the site of attachment in this embodiment may be to a 3' or 5' terminal nucleotide, or to an internal nucleotide, as is more fully described below.

In a preferred embodiment, the conductive oligomer is attached to the ribose of the ribose-phosphate backbone. This may be done in several ways. As is known in the art, nucleosides that are modified at either the 2' or 3' position of the ribose with amino groups, sulfur groups, silicone groups, phosphorus groups, or oxo groups can be made (Imazawa et al., J. Org. Chem., 44:2039 (1979); Hobbs et al., J. Org. Chem. 42(4):714 (1977); Verheyden et al., J. Org. Chem. 36(2):250 (1971); McGee et al., J. Org. Chem. 61:781-785 (1996); Mikhailopulo et al., Liebigs. Ann. Chem. 513-519 (1993); McGee et al., Nucleosides & Nucleotides 14(6):1329 (1995), all of which are incorporated by reference). These modified nucleosides are then used to add the conductive oligomers.

A preferred embodiment utilizes amino-modified nucleosides. These amino-modified riboses can then be used to form either amide or amine linkages to the conductive oligomers. In a preferred embodiment, the amino group is attached directly to the ribose, although as will be appreciated by those in the art, short linkers such as those described herein for "Z" may be present between the amino group and the ribose.

In a preferred embodiment, an amide linkage is used for attachment to the ribose. Preferably, if the conductive oligomer of Structures 13- 15 is used, m is zero and thus the conductive oligomer terminates in the amide bond. In this embodiment, the nitrogen of the amino group of the amino-modified ribose is the "D" atom of the conductive oligomer. Thus, a preferred attachment of this embodiment is depicted in Structure 32 (using the Structure 13 conductive oligomer):

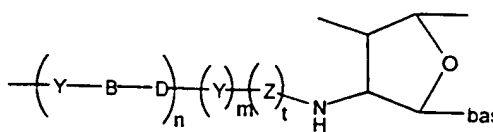
Structure 32



As will be appreciated by those in the art, Structure 32 has the terminal bond fixed as an amide bond.

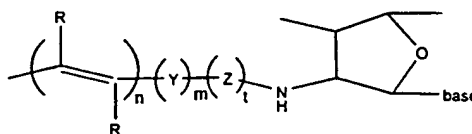
In a preferred embodiment, a heteroatom linkage is used, i.e. oxo, amine, sulfur, etc. A preferred embodiment utilizes an amine linkage. Again, as outlined above for the amide linkages, for amine linkages, the nitrogen of the amino-modified ribose may be the "D" atom of the conductive oligomer when the Structure 3 conductive oligomer is used. Thus, for example, Structures 33 and 34 depict nucleosides with the Structures 13 and 21 conductive oligomers, respectively, using the nitrogen as the heteroatom, although other heteroatoms can be used:

Structure 33



In Structure 33, preferably both m and t are not zero. A preferred Z here is a methylene group, or other aliphatic alkyl linkers. One, two or three carbons in this position are particularly useful for synthetic reasons.

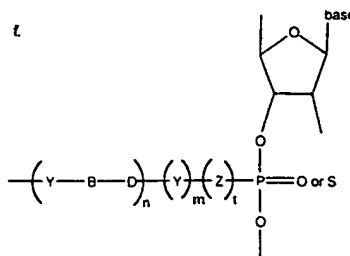
Structure 34



5 In Structure 34, Z is as defined above. Suitable linkers include methylene and ethylene.

In an alternative embodiment, the conductive oligomer is covalently attached to the nucleic acid via the phosphate of the ribose-phosphate backbone (or analog) of a nucleic acid. In this embodiment, the attachment is direct, utilizes a linker or via an amide bond. Structure 35 depicts a direct linkage, and Structure 36 depicts linkage via an amide bond (both utilize the Structure 13 conductive oligomer, although Structure 20 conductive oligomers are also possible). Structures 35 and 36 depict the conductive oligomer in the 3' position, although the 5' position is also possible. Furthermore, both Structures 35 and 36 depict naturally occurring phosphodiester bonds, although as those in the art will appreciate, non-standard analogs of phosphodiester bonds may also be used.

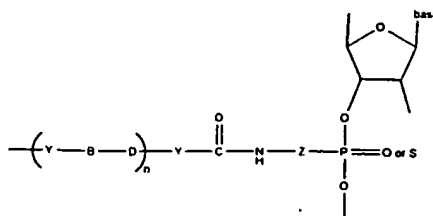
Structure 35



15 In Structure 35, if the terminal Y is present (i.e. m=1), then preferably Z is not present (i.e. t=0). If the terminal Y is not present, then Z is preferably present.

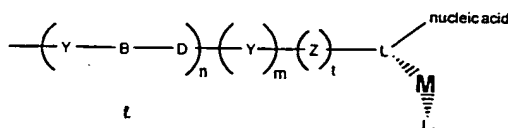
Structure 36 depicts a preferred embodiment, wherein the terminal B-D bond is an amide bond, the terminal Y is not present, and Z is a linker, as defined herein.

Structure 36

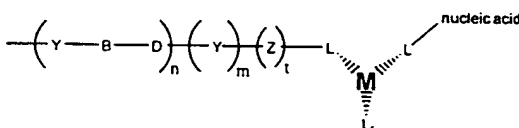


In a preferred embodiment, the conductive oligomer is covalently attached to the nucleic acid via a transition metal ligand. In this embodiment, the conductive oligomer is covalently attached to a ligand which provides one or more of the coordination atoms for a transition metal. In one embodiment, the ligand to which the conductive oligomer is attached also has the nucleic acid attached, as is generally depicted below in Structure 37. Alternatively, the conductive oligomer is attached to one ligand, and the nucleic acid is attached to another ligand, as is generally depicted below in Structure 38. Thus, in the presence of the transition metal, the conductive oligomer is covalently attached to the nucleic acid. Both of these structures depict Structure 13 conductive oligomers, although other oligomers may be utilized. Structures 37 and 38 depict two representative structures:

Structure 37



Structure 38



The use of metal ions to connect the nucleic acids can serve as an internal control or calibration of the system, to evaluate the number of available nucleic acids on the surface. However, as will be appreciated by those in the art, if metal ions are used to connect the nucleic acids to the conductive oligomers, it is generally desirable to have this metal ion complex have a different redox potential than that of the ETMs used in the rest of the system, as described below. This is generally true so as to be able to distinguish the presence of the capture probe from the presence of the target sequence. This may be useful for identification, calibration and/or quantification. Thus, the amount of capture probe on an electrode may be compared to the amount of hybridized double stranded nucleic acid to quantify the amount of target sequence in a sample. This is quite significant to serve as an internal control of the sensor or system. This allows a measurement either prior to the addition of target or after, on the same molecules that will be used for detection, rather than rely on a similar but different control

system. Thus, the actual molecules that will be used for the detection can be quantified prior to any experiment. This is a significant advantage over prior methods.

5 In a preferred embodiment, the capture probe nucleic acids are covalently attached to the electrode via an insulator. The attachment of nucleic acids to insulators such as alkyl groups is well known, and can be done to the base or the backbone, including the ribose or phosphate for backbones containing these moieties, or to alternate backbones for nucleic acid analogs.

10 In a preferred embodiment, there may be one or more different capture probe species on the surface. In some embodiments, there may be one type of capture probe, or one type of capture probe extender, as is more fully described below. Alternatively, different capture probes, or one capture probes with a multiplicity of different capture extender probes can be used. Similarly, it may be desirable (particular in the case of nucleic acid analytes and binding ligands in mechanism-2 systems) to use auxillary capture probes that comprise relatively short probe sequences, that can be used to "tack down" components of the system, for example the recruitment linkers, to increase the concentration of ETMs at the surface.

15 Thus the present invention provides substrates comprising at least one detection electrode comprising monolayers and capture and/or detection probes, useful in target analyte detection systems.

20 The compositions of the invention are generally synthesized as outlined below, generally utilizing techniques well known in the art. As will be appreciated by those in the art, many of the techniques outlined below are directed to nucleic acids containing a ribose-phosphate backbone. However, as outlined above, many alternate nucleic acid analogs may be utilized, some of which may not contain either ribose or phosphate in the backbone. In these embodiments, for attachment at positions other than the base, attachment is done as will be appreciated by those in the art, depending on the backbone. Thus, for example, attachment can be made at the carbon atoms of the PNA backbone, as is described below, or at either terminus of the PNA.

25 The compositions may be made in several ways. A preferred method first synthesizes a conductive oligomer attached to a nucleoside, with addition of additional nucleosides to form the capture probe followed by attachment to the electrode. Alternatively, the whole capture probe may be made and then the completed conductive oligomer added, followed by attachment to the electrode. Alternatively, a monolayer of conductive oligomer (some of which have functional groups for attachment of capture probes) is attached to the electrode first, followed by attachment of the capture probe. The latter two
30 methods may be preferred when conductive oligomers are used which are not stable in the solvents and under the conditions used in traditional nucleic acid synthesis.

In a preferred embodiment, the compositions of the invention are made by first forming the conductive oligomer covalently attached to the nucleoside, followed by the addition of additional nucleosides to form a capture probe nucleic acid, with the last step comprising the addition of the conductive oligomer to the electrode.

- 5 The attachment of the conductive oligomer to the nucleoside may be done in several ways. In a preferred embodiment, all or part of the conductive oligomer is synthesized first (generally with a functional group on the end for attachment to the electrode), which is then attached to the nucleoside. Additional nucleosides are then added as required, with the last step generally being attachment to the electrode. Alternatively, oligomer units are added one at a time to the nucleoside, with addition of
10 additional nucleosides and attachment to the electrode. A number of representative syntheses are shown in the Figures of WO98/20162, expressly incorporated herein by reference.

The conductive oligomer is then attached to a nucleoside that may contain one (or more) of the oligomer units, attached as depicted herein.

- 15 In a preferred embodiment, attachment is to a ribose of the ribose-phosphate backbone. Thus, attachment via amide and amine linkages are possible (see Figures 1 and 2 of WO98/20162). In a preferred embodiment, there is at least a methylene group or other short aliphatic alkyl groups (as a Z group) between the nitrogen attached to the ribose and the aromatic ring of the conductive oligomer. A representative synthesis is shown in Figure 16 of WO98/20162.

- 20 Alternatively, attachment is via a phosphate of the ribose-phosphate backbone. Examples of two synthetic schemes are shown in Figure 4 and Figure 5 of WO98/20162. Although both Figures show attachment at the 3' position of the ribose, attachment can also be made via the 2' position. In Figure 5, Z is an ethylene linker, although other linkers may be used as well, as will be appreciated by those in the art.

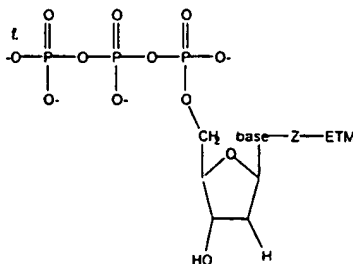
- 25 In a preferred embodiment, attachment is via the base. A general scheme is depicted in Figure 3 of WO98/20162, using uridine as the nucleoside and a phenylene-acetylene conductive oligomer. As will be appreciated in the art, amide linkages are also possible, using techniques well known in the art. In a preferred embodiment, protecting groups may be added to the base prior to addition of the conductive oligomers, as is generally outlined in Figures 10 and 11 of WO98/20162. In addition, the
30 palladium cross-coupling reactions may be altered to prevent dimerization problems; i.e. two conductive oligomers dimerizing, rather than coupling to the base.

Alternatively, attachment to the base may be done by making the nucleoside with one unit of the oligomer, followed by the addition of others.

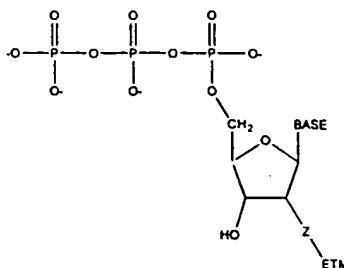
Once the modified nucleosides are prepared, protected and activated, prior to attachment to the electrode, they may be incorporated into a growing oligonucleotide by standard synthetic techniques (Gait, Oligonucleotide Synthesis: A Practical Approach, IRL Press, Oxford, UK 1984; Eckstein) in several ways.

In one embodiment, one or more modified nucleosides are converted to the triphosphate form and incorporated into a growing oligonucleotide chain by using standard molecular biology techniques such as with the use of the enzyme DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, Taq DNA polymerase, reverse transcriptase, and RNA polymerases. For the incorporation of a 3' modified nucleoside to a nucleic acid, terminal deoxynucleotidyltransferase may be used. (Ratliff, Terminal deoxynucleotidyltransferase. In The Enzymes, Vol 14A. P.D. Boyer ed. pp 105-118. Academic Press, San Diego, CA. 1981). Thus, the present invention provides deoxyribonucleoside triphosphates comprising a covalently attached ETM. Preferred embodiments utilize ETM attachment to the base or the backbone, such as the ribose (preferably in the 2' position), as is generally depicted below in Structures 40 and 41:

Structure 40



Structure 41



Thus, in some embodiments, it may be possible to generate the nucleic acids comprising ETMs in situ. For example, a target sequence can hybridize to a capture probe (for example on the surface) in such a way that the terminus of the target sequence is exposed, i.e. unhybridized. The addition of enzyme

and triphosphate nucleotides labelled with ETMs allows the in situ creation of the label. Similarly, using labeled nucleotides recognized by polymerases can allow simultaneous amplification and detection; that is, the target sequences are generated in situ.

5 In a preferred embodiment, the modified nucleoside is converted to the phosphoramidite or H-phosphonate form, which are then used in solid-phase or solution syntheses of oligonucleotides. In this way the modified nucleoside, either for attachment at the ribose (i.e. amino- or thiol-modified nucleosides) or the base, is incorporated into the oligonucleotide at either an internal position or the 5' terminus. This is generally done in one of two ways. First, the 5' position of the ribose is protected with 4',4-dimethoxytrityl (DMT) followed by reaction with either 2-cyanoethoxy-bis-
10 diisopropylaminophosphine in the presence of diisopropylammonium tetrazolide, or by reaction with chlorodiisopropylamino 2'-cyanoethoxyphosphine, to give the phosphoramidite as is known in the art; although other techniques may be used as will be appreciated by those in the art. See Gait, supra; Caruthers, Science 230:281 (1985), both of which are expressly incorporated herein by reference.

15 For attachment of a group to the 3' terminus, a preferred method utilizes the attachment of the modified nucleoside (or the nucleoside replacement) to controlled pore glass (CPG) or other oligomeric supports. In this embodiment, the modified nucleoside is protected at the 5' end with DMT, and then reacted with succinic anhydride with activation. The resulting succinyl compound is attached to CPG or other oligomeric supports as is known in the art. Further phosphoramidite nucleosides are added, either modified or not, to the 5' end after deprotection. Thus, the present invention provides
20 conductive oligomers or insulators covalently attached to nucleosides attached to solid oligomeric supports such as CPG, and phosphoramidite derivatives of the nucleosides of the invention.

The invention further provides methods of making label probes with recruitment linkers comprising ETMs. These synthetic reactions will depend on the character of the recruitment linker and the method of attachment of the ETM, as will be appreciated by those in the art. For nucleic acid
25 recruitment linkers, the label probes are generally made as outlined herein with the incorporation of ETMs at one or more positions. When a transition metal complex is used as the ETM, synthesis may occur in several ways. In a preferred embodiment, the ligand(s) are added to a nucleoside, followed by the transition metal ion, and then the nucleoside with the transition metal complex attached is added to an oligonucleotide, i.e. by addition to the nucleic acid synthesizer. Alternatively, the
30 ligand(s) may be attached, followed by incorporation into a growing oligonucleotide chain, followed by the addition of the metal ion.

In a preferred embodiment, ETMs are attached to a ribose of the ribose-phosphate backbone. This is generally done as is outlined herein for conductive oligomers, as described herein, and in PCT

publication WO 95/15971, using amino-modified or oxo-modified nucleosides, at either the 2' or 3' position of the ribose. The amino group may then be used either as a ligand, for example as a transition metal ligand for attachment of the metal ion, or as a chemically functional group that can be used for attachment of other ligands or organic ETMs, for example via amide linkages, as will be appreciated by those in the art. For example, the examples describe the synthesis of nucleosides with a variety of ETMs attached via the ribose.

In a preferred embodiment, ETMs are attached to a phosphate of the ribose-phosphate backbone. As outlined herein, this may be done using phosphodiester analogs such as phosphoramidite bonds, see generally PCT publication WO 95/15971, or can be done in a similar manner to that depicted in Figures 4 and 5 of PCT US97/20014, where the conductive oligomer is replaced by a transition metal ligand or complex or an organic ETM, as well as is outlined in the Examples.

Attachment to alternate backbones, for example peptide nucleic acids or alternate phosphate linkages will be done as will be appreciated by those in the art.

In a preferred embodiment, ETMs are attached to a base of the nucleoside. This may be done in a variety of ways. In one embodiment, amino groups of the base, either naturally occurring or added as is described herein (see the figures, for example), are used either as ligands for transition metal complexes or as a chemically functional group that can be used to add other ligands, for example via an amide linkage, or organic ETMs. This is done as will be appreciated by those in the art. Alternatively, nucleosides containing halogen atoms attached to the heterocyclic ring are commercially available. Acetylene linked ligands may be added using the halogenated bases, as is generally known; see for example, Tzalis et al., Tetrahedron Lett. 36(34):6017-6020 (1995); Tzalis et al., Tetrahedron Lett. 36(2):3489-3490 (1995); and Tzalis et al., Chem. Communications (in press) 1996, all of which are hereby expressly incorporated by reference. See also the figures and the examples, which describes the synthesis of metallocenes (in this case, ferrocene) attached via acetylene linkages to the bases.

In one embodiment, the nucleosides are made with transition metal ligands, incorporated into a nucleic acid, and then the transition metal ion and any remaining necessary ligands are added as is known in the art. In an alternative embodiment, the transition metal ion and additional ligands are added prior to incorporation into the nucleic acid.

Once the nucleic acids of the invention are made, with a covalently attached attachment linker (i.e. either an insulator or a conductive oligomer), the attachment linker is attached to the electrode. The method will vary depending on the type of electrode used. As is described herein, the attachment

linkers are generally made with a terminal "A" linker to facilitate attachment to the electrode. For the purposes of this application, a sulfur-gold attachment is considered a covalent attachment.

5 In a preferred embodiment, conductive oligomers, insulators, and attachment linkers are covalently attached via sulfur linkages to the electrode. However, surprisingly, traditional protecting groups for use of attaching molecules to gold electrodes are generally not ideal for use in both synthesis of the compositions described herein and inclusion in oligonucleotide synthetic reactions. Accordingly, the present invention provides novel methods for the attachment of conductive oligomers to gold electrodes, utilizing unusual protecting groups, including ethylpyridine, and trimethylsilylethyl as is depicted in the Figures. However, as will be appreciated by those in the art, when the conductive
10 oligomers do not contain nucleic acids, traditional protecting groups such as acetyl groups and others may be used. See Greene et al., supra.

This may be done in several ways. In a preferred embodiment, the subunit of the conductive oligomer which contains the sulfur atom for attachment to the electrode is protected with an ethyl-pyridine or trimethylsilylethyl group. For the former, this is generally done by contacting the subunit containing the sulfur atom (preferably in the form of a sulfhydryl) with a vinyl pyridine group or vinyl trimethylsilylethyl group under conditions whereby an ethylpyridine group or trimethylsilylethyl group is added to the sulfur atom.
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This subunit also generally contains a functional moiety for attachment of additional subunits, and thus additional subunits are attached to form the conductive oligomer. The conductive oligomer is then attached to a nucleoside, and additional nucleosides attached. The protecting group is then removed and the sulfur-gold covalent attachment is made. Alternatively, all or part of the conductive oligomer is made, and then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. The conductive oligomer is then attached to a nucleoside, and additional nucleosides attached. Alternatively, the conductive oligomer attached to a nucleic acid is made, and
20 then either a subunit containing a protected sulfur atom is added, or a sulfur atom is added and then protected. Alternatively, the ethyl pyridine protecting group may be used as above, but removed after one or more steps and replaced with a standard protecting group like a disulfide. Thus, the ethyl pyridine or trimethylsilylethyl group may serve as the protecting group for some of the synthetic reactions, and then removed and replaced with a traditional protecting group.
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30 By "subunit" of a conductive polymer herein is meant at least the moiety of the conductive oligomer to which the sulfur atom is attached, although additional atoms may be present, including either functional groups which allow the addition of additional components of the conductive oligomer, or